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Original Submission

GRAS Notification

for

Hayashibara Trehalose

Submitted by

Hayashibara International Inc.

2201 Civic Circle, Suite 719
Amarillo, Texas USA
79109

May 3,2000

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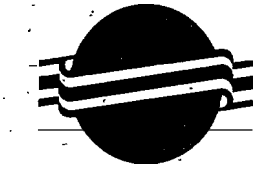

2201 Civic Circle, Suite 719
Amarillo, Texas 79109
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HAYASHIBARA INTERNATIONAL INC.

May 8, 2000

Dr. Laura Tarantino
Acting Director
Office of Premarket Approval
Center for Food, Safety and Nutrition HFS 200
Food and Drug Administration.
200 C S1. SW
Washington, DC 20204

Dear Dr. Tarantino:

In accordance with the proposed rule for Substances Generally Recognized as Safe, which was published in the *FederalRegister* at Vol. 62, No. 74 on April 17, 1997, Hayashibara International Inc. (Hayashibara) of Amarillo, Texas would like to submit a notice of a claim that the use of Hayashibara trehalose as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS. Trehalose is a naturally occurring sugar, which is commercially manufactured from cornstarch, using food grade ingredients in accordance with current Good Manufacturing Practices. Analytically, trehalose is a nearly pure source of carbohydrate (≥ 99.0% on a dry weight basis).

Trehalose is a disaccharide composed of two glucose molecules. Metabolically, trehalose is hydrolyzed to glucose by the enzyme trehalase in a manner similar to the digestion of other disaccharides. Nutritionally, it provides 3.6 calories per gram, and is approximately 45% as sweet as sucrose.

A GRAS Report in support of the safe use of trehalose in foods was prepared by Hayashibara, and was reviewed on November 7, 1999 by a panel of experts qualified by training and experience to assess the safety of food ingredients. The experts concurred with Hayashibara's determination that trehalose is safe for general use in foods. A copy of the expert opinion is attached to this notice.

On April 19, 2000 Hayashibara personnel presented the salient points of the GRAS Report, and the conclusions of the expert panel to officials in the Center for Food Safety, and Applied Nutrition (CFSAN). Comments from that meeting, and an earlier informational meeting with Center personnel, held July 9,1999 have been incorporated into this notice

of a claim for premarket exemption. This notification is based on a GRAS determination under proposed §170.36.

Hayashibara has prepared a notification document in triplicate, which accompanies this letter. A fourth copy is enclosed which is intended for distribution to the United States Department of Agriculture at FDA's discretion. The company would appreciate notice of the receipt of this document, and looks forward to any comments the agency would care to make on the notification.

Thank you very much for your attention to this matter. If you have any questions regarding the content of the notification, please contact Dr. Alan Richards at (806) 468-7711.

Sincerely,

Alan B. Richards, Ph.D.
Vice President

CC: Mr. Katsuaki Hayashibara, Hayashibara Company, Ltd.
Ms. Susanne Dvorak, Cargill
Dr. Arthur Lippman, CFSAN
Dr. Linda Kahl, CFSAN
Ms. Lee Dexter, Technical consultant

Claim

Hayashibara International Inc.
GRAS Notification

Introduction

Trehalose is a disaccharide found in nature in thousands of organisms, and has been consumed by humans as part of the diet for thousands of years. It consists of two glucose molecules linked in an a,a 1→1 bond. The Hayashibara Company, Ltd. (Hayashibara) of Okayama, Japan invented a unique process to manufacture trehalose in a cost-effective manner. This has made trehalose available to be used as a food ingredient. This Notification document contains the information required in proposed 170.36 to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination. The document is being submitted by Hayashibara International Inc., of Amarillo, Texas which is a wholly-owned subsidiary of Hayashibara. Both companies will be referred to as "Hayashibara" in this Notification, unless a specific distinction is necessary.

In compliance with 21 CFR § 170.30, Hayashibara determined that Hayashibara trehalose can be considered GRAS when used in accordance with current Good Manufacturing Practices. Hayashibara wishes to voluntarily notify the Center for Food Safety and Applied Nutrition (CFSAN) of that determination, and according to proposed § 170.36, the company is submitting the following GRAS exemption claim.

Hayashibara International, Inc. has prepared an eighteen-volume GRAS Report, which forms the basis for the information found in this notification. The company also commissioned a panel of experts (Expert Panel), qualified by scientific training and experience to assess the safety of food ingredients, who critically evaluated the trehalose GRAS Report as well as other data and information relevant to the use and safety of this ingredient. In a meeting held on November 7, 1999, the panel concurred with the company's determination that Hayashibara trehalose can be considered generally recognized as safe for general use in food. Based on the data and information contained in the Report and the opinion of the expert panel (which is attached to this notification), Hayashibara explicitly accepts responsibility for the GRAS determination of Hayashibara trehalose.

Section I. GRAS Exemption Claim

Hayashibara International, Inc. hereby notifies the U.S. Food and Drug Administration that the use of Hayashibara trehalose as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Hayashibara has determined that such use is GRAS.

4. Notifier:

Hayashibara International Inc.
2201 Civic Circle, Suite 719
Amarillo, Texas, USA 79109
Telephone: (806) 468-7711
Fax: (806) 468-7712

5. Common or Usual Name:

Trehalose

3. Applicable Conditions of Use:

Applications for trehalose include general use in foods as a multiple-use direct additive. The ingredient should be used under the conditions of current Good Manufacturing Practice. In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (0) (1-32). Applications for trehalose are covered under several of the following terms as listed under 21 CFR §170.3 (0).

(4) "Colors and coloring adjuncts": Substances used to impart, preserve, or enhance the color or shading of a food, including color stabilizers, color fixatives, color-retention agents, etc.
(11) "Flavor enhancers": Substances added to supplement, enhance or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.
(16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.
(21) "Nutritive sweeteners": Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

Section I Notification Claim (Continued)

(28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.
(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
(32)"Texturizers": Substances which affect the appearance or feel of the food.

6. Basis of the GRAS Determination

The basis of the GRAS determination for Hayashibara trehalose was the use of scientific procedures.

7. Availability of Data and Information and Key to References

The data and information that are the basis of the GRAS determination for Hayashibara trehalose will be available at the address of the notifier listed above. However, throughout this Notification, citations to the published literature, which were included in the 18-volume GRAS Report are denoted as follows: [Author *(et al),* Year, Tab (number) Volume (number)]. In order to facilitate review of this document a complete list of references from the trehalose GRAS Report is included in Appendix 2 as a key. Recently identified references, which were not included in the GRAS Report are shown between parentheses ( ) within the text of this document and given in a standard bibliographic form.

6. Signature of an official for Hayashibara International, Inc.

Official for Hayashibara International Inc. Date

[redacted] May 8, 2000

Dr. Alan Richards. Vice President

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Notification Section II

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Section II. Chemical Identity

A. Common or Usual Name and Identity

1. a,a-trehalose (dihydrate crystalline trehalose)

2. Trehalose is the preferred common or usual name for Hayashibara's product.

B. Formal Names (IUPAC or Chemical Abstracts Names)

1. a,a-Trehalose

2. a-D-glucopyranosyl a-D-glucopyranoside

C. Synonyms, Other Common Names, Tradenames

1. Trademark: [redacted]™ and [redacted]™. These are registered trademarked names associated with Hayashibara's trehalose.

2. Hayashibara trehalose

3. There are no other common or usual names used in commerce. The term "mushroom sugar" and "mycose" was used for trehalose in some published literature prior to 1970.

D. Chemical Formulae, Structures and Molecular Weights

1. Empirical Formula

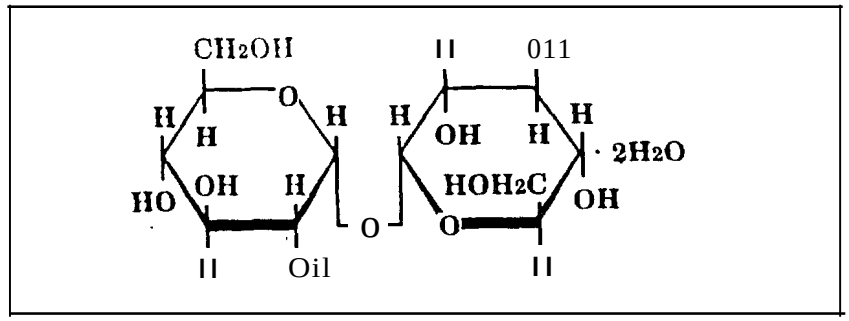
$C_{12}H_{22}O_{11}\cdot 2H_2O$ Trehalose dihydrate

$C_{12}H_{22}O_{11}$ Trehalose anhydrous

Section II. Chemicalldentitv (Continued)

D. Chemical Formulae, Structures and Molecular Weights (continued)

2. Structural Formula



3. Molecular Weight

378.34 {dihydrate)
342.31 (anhydrous)

E. Chemical Abstracts Service Registry Number (CAS Registry No.)

1. CAS Number 99-20-7 (anhydrous)
2. CAS Number 6138-23-4 (dihydrate)

F. Quantitative Composition of Hayashibara trehalose

1. Product Identity

The Hayashibara trehalose product is a non-reducing disaccharide sugar produced by the enzymatic modification of starch. The trehalose product is composed of ≥ 98% trehalose, 0.5% glucose and two trisaccharides composed of only glucose. One trisaccharide comprises 0.3% of the product and is composed of a-D-maltosyl u-D-glucoside. The second trisaccharide comprises 0.1 % of the product and is composed of a-D-isomaltosyl a-D-glucoside. No tetra or larger saccharides are present. The Hayashibara production process results in trehalose dihydrate, which contains 9.5% water. Residue on ignition does not exceed 0.05%. The pH of a 30% trehalose solution is 4.5 to 6.5. Heavy metals and arsenic are below the level of detection. Microbial limits have been set to meet food grade specifications (Table 1).

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Section II. Chemical Identity (Continued)

TABLE 1
FINAL PRODUCT SPECIFICATIONS OF HAYASHIBARA TREHALOSE

Variables	Specifications
Purity (Trehalose)	≥ 98.0%
Appearance	whitish crystalline powder (dihydrate)
Coloration of the Solution	≤ 0.100
Turbidity of the Solution	≤ 0.050
pH (30% Solution)	4.5 - 6.5
Loss on Drying	≤ 1.5%
Residue on Ignition	≤ 0.05%
Lead	≤ 0.1 ppm
Arsenic (as AS203)	< 2 ppm
Viable Count	≤ 300 CFU/g
Coliform Organisms	negative
Yeast and Mold	≤ 100 CFU/g

2. Analysis of Five Lots of Hayashibara trehalose

Five (5) lots of Hayashibara trehalose manufactured from November 16, 1996 to March 3, 1997 showed that the loss of moisture from a standardized drying method was less than 0.15%. There was no detectable residue on ignition and the pH of the product ranged from 5.5 to 5.75. The coloration of the products measured by spectrophotometer at 420 nm and 720 nm ranged from 0.025 to 0.042. Turbidity of a 49.25% solution measured at 720 nm ranged from 0.007 to 0.015. The purity as percent trehalose ranged from 99.05% to 99.27%. Heavy metals (as Pb) were below the level of detection at less than 1 μg/g. Arsenic was found to be less than 2 μg/g. Lead content was less than 1 μg/g. Viable plate counts were less than 5 CFUs/g. Coliform organisms were negative and yeast and mold counts were less than 16 (Table 2). The raw data sheets for certain specifications are included below [Hayashibara, 1997, Vol 6 Tab 64].

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TABLE 2
SUMMARY OF ANALYTICAL RESULTS OF FIVE TREHALOSE LOTS

Variables	Lot No. 611161		Lot No. 612141		Lot No. 701091		Lot No. 702171		Lot No. 703031		X'±SD
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2^{0a}	
Purity (%)	99.26	9912	9920	99.25	99.19	9927	99.10	9905	9916	9913	99 17±0 06
Coloration	0025	0025	0041	0.039	0037	0036	0034	0.034	0042	0040	a 035±0 00 1
Turbidity	0007	0007	0016	0014	0.015	0013	0.015	0016	0013	0014	O013±0 00 3
PH (30% Solution)	5.65	563	5.58	5.52	573	5.75	556	555	555	5.60	5. J6±0 08
Residue on Ignition *(Yo)*	000	000	000	0.00	0.00	0.00	a00	000	000	0.00	-
Loss on Drying (%)	010	0.09	0.09	012	0.11	012	0.13	0.12	011	011	O 1110.01
Heavy Metals (µg/g)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	BOLL
Arsenic (µg/g)	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	BOL
Lead (µg/g)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	80L
Viable Count (CFUlg)	0	0	4	3	1	3	0	2	5	3	21:1:1.7
Coliform Organisms (CFUlQ)	Negative	negative	negative	Negative	negative	negative	negative	negative	negative	negative	8DL
Yeast and Mold	1	1	a	0	4	6	3	5	16	9	*4 5t4* 9
Production Date	November 16, 1996		December 14, 1996		January 9. 1997		February 17, 1997		March 3, 1997		

IThe duplicate results from each lot were averaged and the averages were used to calculate the mean and standard deviation (SD: n=s).
2SDL - Selow detectable limits.

Section II. Chemicalldentitv (Continued)

G. Manufacturing Process

1. History

Historically, trehalose has been produced in the laboratory and in small scale batches using methods that include; extraction from yeast and other natural sources, chemical synthesis, microbial fermentation, enzymatic conversion from maltose, and most recently transgenic technology [Birch. 1965. Vol 4 Tab 19; Cabib and Leloir, 1957, Vol 4 Tab 28, Steiner and Cori, 1935, Vol 12 Tab 124; Koch and Koch. 1925. Vol 7 Tab 76; Murao *et al.,* 1985, Vol 8 Tab 25; Leibowitz, 1944. Vol 7 Tab 80; Stewart *et al.•* 1950, Vol 12 Tab 126; Lemieux and Bauer, 1953. Vol 7 Tab 81; Sugimoto, 1995, Vol 12 Tab 129; Suzuki *etal.,* 1969, Vol 12 Tab 130; and Robinson and Morgan, 1928, Vol 9 Tab 111]. However, none of these methods have been successful in producing trehalose on a large scale at a cost which would make it commercially viable for sale to the food industry. Until recently trehalose was produced in marginal quantities either by extraction from yeast or by bacterial fermentation.

Many scientists have suggested that the production of trehalose by an enzymatic synthesis ;s impractical although production of trehalose by bacteria and other organisms is quite common in nature [Birch, 1965, Vol 4 Tab 19; Elbein, 1974, Vol 5 Tab 49; and Sugimoto. 1995. Vol 12 Tab 129]. One reason for the skepticism is the basic nature of the natural synthetic process. While there appear to be several enzymatic systems, one of the best characterized includes the following two enzymes:

- trehalose 6-phosphate synthetase synthesizes trehalose 6-phosphate from glucose 6-phosphate, and UDP glucose.
- trehalose 6-phosphatase converts trehalose 6-phosphate into trehalose by removing the phosphate group.

[Elbein, 1974. Vol 5 Tab 49. Maruta, et al., 1995, Vol 8 Tab 89, Matula, et al., 1971, Vol 8 Tab 92, Nakada et al., 1995a, Vol 9 Tab 96 and 1995b. Vol 9 Tab 97, Sugimoto, 1995, Vol 12 Tab 129, and Tsusaki et aL, 1997, Vol 12 Tab 134].

This process, however. relies on costly and/or complex intermediates, and therefore has not been considered suitable for commercial production.

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Section II. Chemical Identity (Continued

All of the previous methods for producing trehalose have had low production efficiencies and high selling prices. Previous prices for commercial trehalose have ranged from 20 to 30 thousand Japa"nese Yen per kilogram (-110 ¥ =$1 USD). Hayashibara, using its expertise with enzymes, has recently developed a process, which allows trehalose to be produced for less than 500 Japanese Yen per kilogram [Sugimoto, 1995, Vol 12 Tab 129].

2. Hayashibara Enzymes

Hayashibara scientists screened thousands of isolates of soil bacteria for the presence of enzymes capable of producing trehalose. These bacteria were often symbionts with leguminous plants that appeared to have potential to produce commercial quantities of trehalose. They are members of the genera *Pime/obaeter, Pseudomonas, Rhizobium* and *Arthrobaeter* [Maruta, et al., 1995, Tab 89 Vol 8, and Nakada, *et a/.,* 1995a and 1995b, Tabs 96 and 97 Vol 9]. Two particular groups of bacteria were identified, which can be distinguished based on the substrate they require for trehalose production. The first group of bacteria produces trehalose directly from starch, and use two novel enzymes, maltooligosyl-trehalose synthase and maltooligosyl-trehalose trehalohydrolase
[Nakada, *et a/.,* 1995a, Vol 9 Tab 96]. There are also bacteria which produce trehalose from maltose using a single enzyme, trehalose synthase [Nishimoto, *et a/.,* 1995, Vol 9 Tab 104]. All three enzymes have been characterized in the published literature.

For commercial production, Hayashibara uses the two-enzyme system with other common food grade enzymes to produce trehalose from cornstarch. Starch is relatively inexpensive and the enzymes can process starch from other sources. The mechanism be which the two enzymes convert starch to trehalose is explained below and in Tsusaki, et al. [Tsusaki, et al., 1997, Vol 18, Tab 148].

The enzymes utilized in the Hayashibara process are derived from non-pathogenic soil organisms. The specific enzymes producing trehalose are derived from a bacterium in the genus *Arthrobaeter* (originally designated as *Arthrobaeter sp.* 036). [Nakada, et al., 1995a & b, Vol 9 Tab 96 & 97]. This organism has now been speciated as *Arthrobaeter ramosus.* Hayashibara scientists have

Section II. Chemical Identity (Continued)

developed enzymatic and Elisa assays for the enzymes, which show that there is no detectable enzyme activity or residue remaining in the final product [Hayashibara International, 1997, Vol 18 Tab 148].

Hayashibara International commissioned Dr. Michael Pariza, Director of the Food Research Institute of the University of Wisconsin, to write an expert opinion on the safety of the novel enzymes used in the Hayashibara production process. That expert opinion was considered by

the Expert Panel, and can be found in the GRAS Report [Tab 150 Vol 18]. Additionally, Mr. Cleve Denny, a member of the Expert Panel and a known expert on microbial safety of foods wrote an opinion. Copies of both letters are attached to this Notification as Appendix 3.

here thru p15 confidential

Section II. Chemical Identity (Continued)

Section II. Chemical Identity (Continued)

Section II. Chemical Identity (Continued)

Section II. Chemical Identity (Continued)

Figure 2

Section II. Chemical Identity (Continued)

Section II. Chemicalldentitv (Continued)

H. Characteristic Properties of Trehalose

1. Introduction

Trehalose is a stable non-reducing disaccharide with two glucose molecules linked in an a, a-1,1 configuration. For comparison, maltose is a reducing disaccharide with 2 glucose molecules linked in an α, a-1,4 configuration [Birch, 1965, Vol 4 Tab 19; Sugimoto, 1995, Vol 12 Tab 129]. Trehalose exists naturally in plants, animals and microorganisms, and has long been consumed by humans as a component of mushrooms, baker's and brewer's yeasts, seaweeds and such invertebrates as lobsters [Elbein, 1974, Vol 5 Tab 49; Wyatt. and Kalf, 1957, Vol 18 Tab 143]. Trehalose is believed to playa key role in the preservation of biomembranes and in the revival of certain biological functions following desiccation or freezing [Roser, 1991a, Vol 9 Tab 113; Roser, 1991b, Vol 9 Tab 114; Sugimoto, 1995, Vol 12 Tab 129]. Many applications have been purposed for the unique characteristics of trehalose. [Colaço and Roser; 1995, Vol 4 Tab 35; Colaço, *et al.,* 1992, Vol 4 Tab 36; MacDonald and Lanier, 1991, Vol 8 Tab 84; and Sugimoto, 1995, Vol 12 Tab 129].

2. Long Term Stability of Hayashibara Trehalose

In order to determine the integrity of their product over its intended storage life, Hayashibara has conducted analyses of specification variables on trehalose, which has been stored from 0-12 months. The product was tested at 0, 1,3,6,9 and 12 months. The results showed that the percent purity of the product increased slightly from 99.1 to 99.3% over the period, which is most likely due to concurrent loss of moisture. The product lost moisture after the first month from 0.06% to 0.31%. There was no residue on ignition after the product was initially produced. pH remained relatively stable between 5.5 and 6.1 during the period. Colorization also remained stable from 0.018 to 0.025. Turbidity was also stable from 0.002 to 0.007. Heavy metals and arsenic were below limits of detection after the production period. Therefore, testing for these two variables was discontinued during the remaining sample periods. Viable count of microorganisms dropped over the period from 17 CFU/g to 0. Coliform organisms remained negative over the storage period, and yeast and mold counts dropped from 19 to 1 over the twelve months.

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Section II. Chemicalldentitv (Continued)

These results confirmed that the quality control measures Hayashibara has in place are sufficient to maintain the integrity of the product over a lengthy storage period [Table 3].

Test sample: Hayashibara trehalose Lot No. 512061 (production date: December 1995)

Packaging mode: 3-layer Kraft paper bag (1 layer is polyethylene);
Net weight. 20kg

Storage conditions: Warehouse at 25° C constant temperature

Sampling: 100 g each time from the same bag

Dates of analyses: 0, 1, 3, 6, 9, and 12 months

Results

Table 3
Hayashibara trehalose Stability Study

Figure 3
Crystalline Form of Hayashibara Trehalose

CRYSTAL FORM OF HAYASHIBARA TREHALOSE

[Material] HAYASHIBARA Trehalose (crystalline dihydrate)

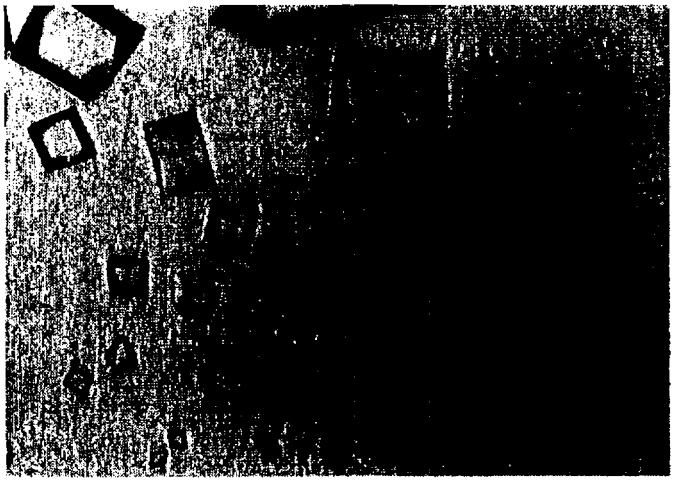
[Equipment and Apparatus]

Microscope: BH-2 (Olympus Kogaku Co.)

[Conditions] Test sample: Crystalline trehalose (Purity: 100.0%)
Magnification: x50

[Method] After trehalose is dissolved in water by heating at 50°C, the crystalline form of trehalose is observed microscopically.

[Results] HAYASHIBARA trehalose is colorless and has a prismatic crystalline form.



HAYASHIBARA trehalose (x50)

[Results] Stability of HAYASHIBARA Trehalose

HAYASffiBARA Trehalose in solution is stable for 1 year.

Storage period (months)	25°C pH	Color value	Turbidity	Residual ratio
0	6.80	0.000	0.002	100
1	5.31	0.000	0.004	100
2	5.05	0.000	0.000	100
3	5.37	0.000	0.000	100
4	5.32	0.000	0.000	
5	5.42	0.000	0.000	
6	5.36	0.000	0.000	99
9	5.16	0.000	0.000	100
12	5.27	0.000	0.000	101

Storage period	37°C			ratio
0	6.80	0.000	0.002	100
1	5.46	0.000	0.000	100
2	5.21	0.000	0.000	100
3	5.32	0.000	0.000	100
4	5.26	0.005	0.000	
5	5.48	0.003	0.000	
6	5.13	0.002	0.000	100
9	5.10	0.000	0.005	100
12	5.15	0.000	0.000	100

Section II. Chemicalldentitv (Continued)

3. Physical and Chemical Structure of Trehalose

The physical and chemical structure of a,a-trehalose have been assayed using various preparations of trehalose. Some of these variables are present in Table 4 below.

Section II. Chemicalldentitv (Continued)

Table 4
STRUCTURE OF TREHALOSE
(CRYSTALLINE DIHYDRATE)

Composition of elements[1]	Theoretical / Actual C 38.10% / 37.87% H 6.93% / 6.93% O 54.97% / 54.97%
Chemical formula[1]	$C_{12}H_{22}O_{11}\bullet 2H_2O$
Molecular weight[1]	378.33
Melting point[1]	Dihydrate 97°C Anhydrous 210.5°C
Specific optical rotation[1]	$[a]_D^{20}$ + 199°
Heat of fusion[1]	Dihydrate 57.8 kJ/mol Anhydrous 53.4 kJ/mol
Crystal system[1]	Rhomboid crystal
Cell parameters[2]	a = 12.23A b = 17.89 A c = 7.66 A
Orthorhombic cell[2]	$P2_1\ 2_1\ 2_1$
Units per cell[2]	4
Theoretical density[2]	*1.511 (g/cm^3)*
Actual density	*1.512 (g/cm^3)*

1These data were generated using Hayashibara trehalose.
2These data were generated using other preparations of trehalose [Brown *et a/.,* 1972,Vo14 Tab 26 and Taga *at a/.,* 1972, Vol 12 Tab 132].

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Section II. Chemlcalldentltv (Continued)

4. X-Ray Refraction of Trehalose Powder

An x-ray diffractometer was used to measure the x-ray refraction image of dihydrate and anhydrous crystalline trehalose. Results showed that the dihydrate form produced more variable peaks with greater intensities than did the anhydrous form.

Material: Hayashibara trehalose (crystalline dihydrate)

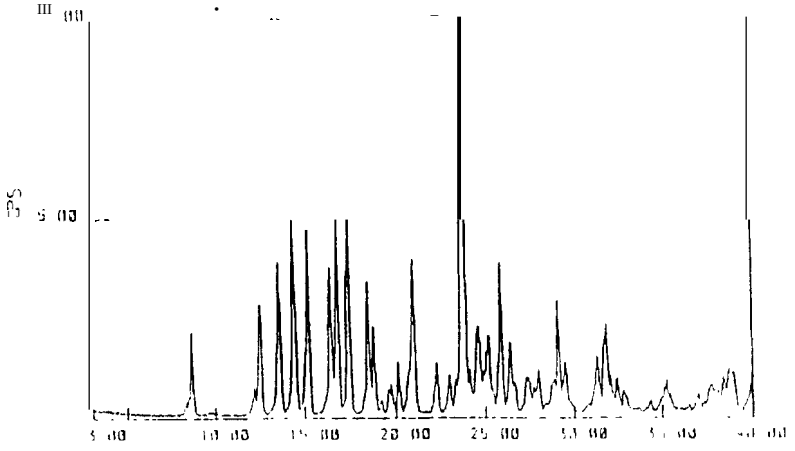
Equipment and Apparatus: X-Ray Diffractometer: Geiger Flex RAD=28 (Rigaku Denki Co.)

Method: The x-ray refraction of trehalose was analyzed by a powder method using an x-ray diffractometer.

Conditions: Test Sample: Crystalline trehalose (Purity: 100.0%)
X-ray beam: Cu. Ka (monochromator)
Voltage: 40 kV
Current: 30 rnA

Results: Results shown on the next page identify fewer peaks at less intensity in the anhydrous trehalose than for the dihydrate form [Figure 4].

X-ray refraction image of dihydrate crystalline trehalose



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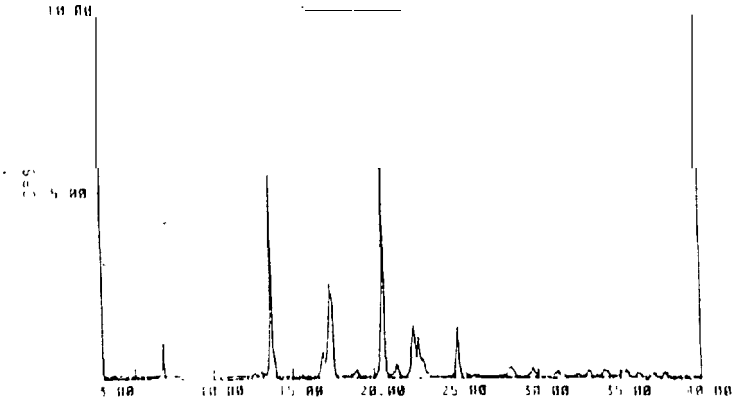
Section II. Chemicalldentitv (Continued)

Figure 4

X-ray refraction image of anhydrous crystalline trehalose



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5. Melting Point ;

Summary: The melting point of Hayashibara trehalose in both the anhydrous and dihydrate forms was measured in a melting point testing apparatus. Results showed that the anhydrous form melted at 210.5°C, while the dihydrate form melted at 97°C. The actual result of the melting point for the anhydrous form was 7.5°C higher than that reported in the literature.

Material: Hayashibara trehalose (crystalline dihydrate)

Equipment and Apparatus: Melting point apparatus: MP-21 (Yamato Kagaku Co.)

Method: The melting point of trehalose is measured on a melting point apparatus by placing the test sample powder in the measurement tube.

Conditions: Test sample: Crystalline trehalose (Purity 100.0%)

Results: Results showed that the anhydrous form of trehalose had a higher melting point by 113.5°C than did the dihydrate form. The values obtained for the dihydrate and anhydrous forms are compared to those found in published literature in the table below.

Hayashibara trehalose (crystalline dihydrate): 97.0°C (97°C*)

Section II. Chemical Identity (Continued)

Hayashibara trehalose (crystalline anhydrous): 210.5°C (203°C*)

**Values listed in the "Dictionary of Chemistry" published by Kyoritsu Publishing Co.*

6. Heat of Fusion

Summary: A differential scanning calorimeter was used to measure the heat of fusion of dihydrate crystalline trehalose. Results showed that the heat of fusion was 57.8 kJ/mol for the dihydrate form.

Material: Hayashibara trehalose (crystalline dihydrate)

Equipment and Apparatus: Differential Scanning Calorimeter: DSC 8230 (Rigaku Denki Co.)

Method: Fusion heat is measured by applying the powdered sample to the differential scanning calorimeter.

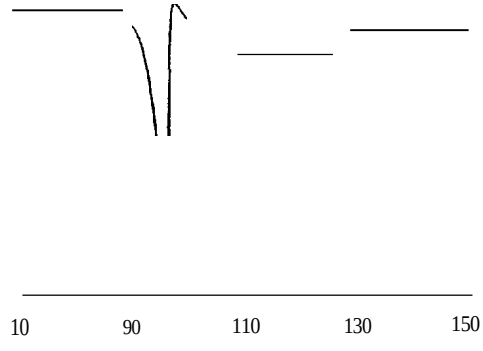
Conditions: Test sample: Crystalline trehalose (Purity: 100.0%)

Results: The fusion heat of Hayashibara crystalline dihydrate trehalose was found to be 57.8 kJ/mol, and the fusion heat of Hayashibara anhydrous crystalline trehalose was 53.4 kJ/mol, as shown below [Figure 5].

Section ll. Chemical Identity (Continued)

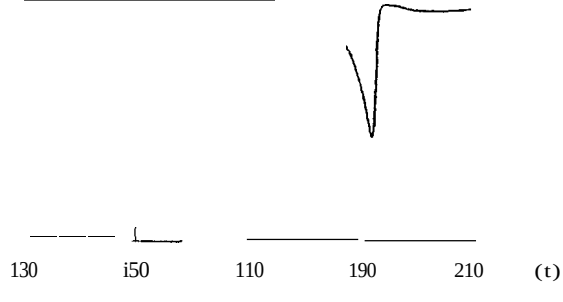
Figure 5
Hayashibara Trehalose
Fusion Heat

The fusion heat of HAYASHIBARA dihydrate cr)'stalline trehalose is 57.8 *kllmol*



DSC Cunoe

The fusion heat of HAYASHIBARA anhydrous crystalline trehalose is 53.4 *kllmol*



DSC Curve

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Section II. Chemicalldentitv (Continued)

7. Specific Optical Rotation

Summary: A polarimeter was used to measure the specific rotation of the dihydrate form of the Hayashibara trehalose. Results of the experiment showed that the specific rotation of the crystals was $[\alpha]_D^{20}$: *+199°.*

Material: Hayashibara trehalose (crystalline dihydrate)

Equipment and Apparatus: Polarimeter: SEPA-300 (Horiba Co.)

Method: Test solution containing 5% *(w/v)* of the test sample is tested by optical rotation at 20°C.

Conditions: Test Sample: Crystalline trehalose (Purity 100.0%)

Results: Results of the polarimeter measurements showed that the trehalose crystals have a specific rotation $[\alpha]_D^{20}$: *+199°* (c=5).

8. Trehalose Absorption Spectra

Summary: An infrared and a standard spectrometer were used to measure the absorption spectra for anhydrous and dihydrate crystalline trehalose. No specific absorption spectra were observed in the UV and visible ranges, while spectra in the infrared range yielded strong peaks at approximately 900 nm and between 3200 and 3500 nm for both materials.

Material: Hayashibara trehalose (crystalline dihydrate)

Equipment and Apparatus: Spectrophotometer: UV-160 (Shimazu Seisakusho Co.) (UV and Visible Absorption Spectrum) Infrared Spectrophotometer: Type 210 (Hitachi) (Infrared Range Absorption Spectrum)

Method: UV and"visible range absorption spectra were measured using a solution containing 2% (wlv) of the test sample. Spectrophotometer readings were taken at wavelengths of 200 to 800 nm.

Infrared range absorption spectra: Two (2) mg of the test sample and 200 mg of dried KBr were mixed, ground, and homogenized to

Section II. Chemicalldentitv (Continued)

prepare tablets. The tablets were used to measure the absorption spectrum on an infrared spectrophotometer.

Conditions: Test Sample: Crystalline trehalose (Purity 100.0%)

Results: Results showed that no specific absorption was noted for either the anhydrous or dihydrate forms of the material in the visible or UV ranges. Infrared spectra of the two materials yielded strong peaks at approximately 900 nm and between 3200 and 3500 nm.

UV range absorption spectrum-No specific absorption was noted in either test sample.

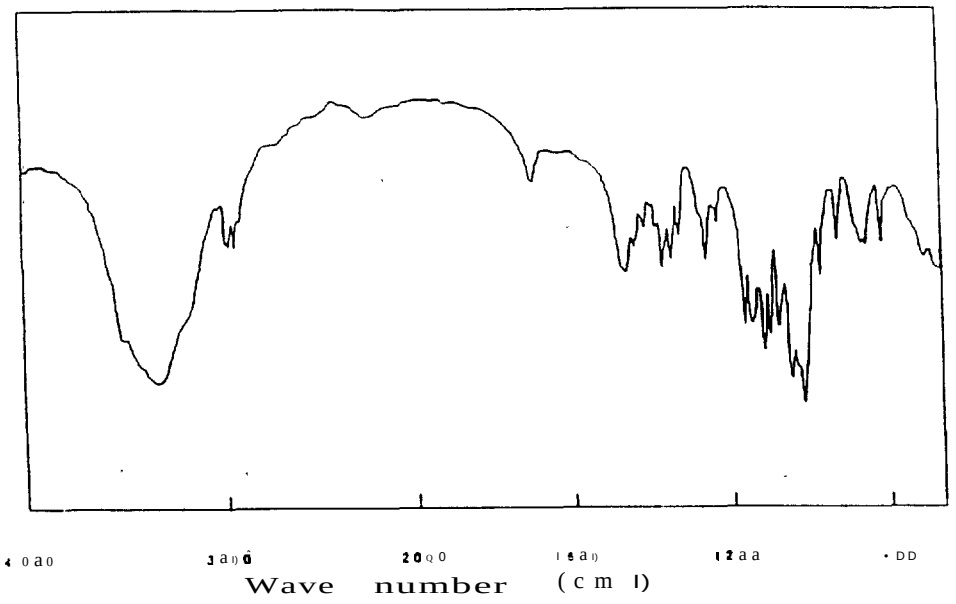
Visible range absorption spectrum-No specific absorption was noted in either test sample.

Infrared range absorption spectrum-[See spectra Figure 6].

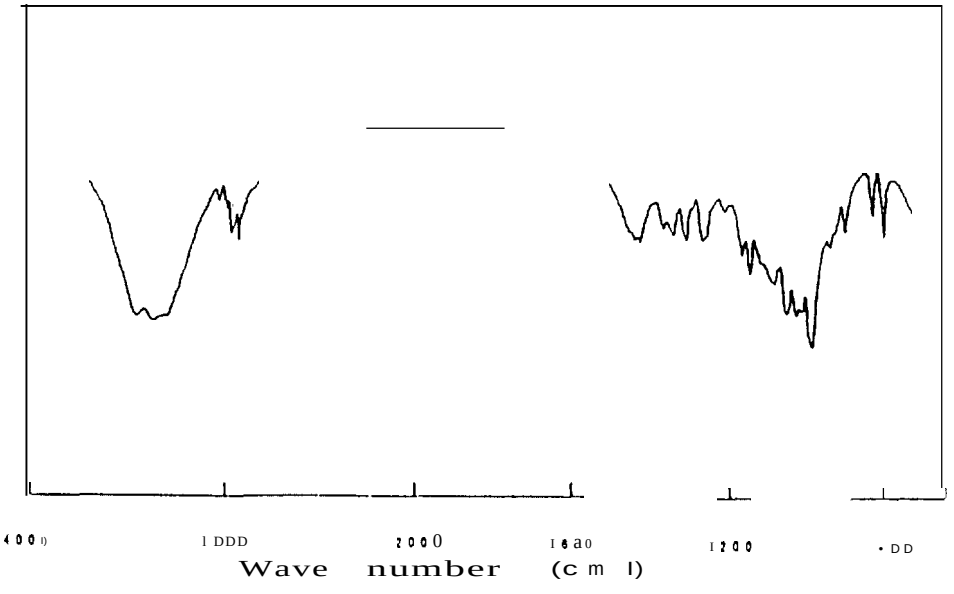
Section II. Chemicalldentitv (Continued)

Figure 6
Hayashibara Trehalose Absorption Spectra

Infrared Range Absorption Spectrum of Trehalose (crystalline dihydrate)



Infrared Range Absorption Spectrum of Trehalose (crystalline anhydrous)



Section II. Chemicalldentitv (Continued)

9. Liquid Chromatogram Analysis

Summary: A sample of Hayashibara trehalose was tested via a liquid chromatogram technique to determine purity. Results of the experiment showed that the sample was comprised of 100% trehalose with no detectable impurities.

Material: Hayashibara trehalose (Purity: 100%)

Equipment and Conditions:

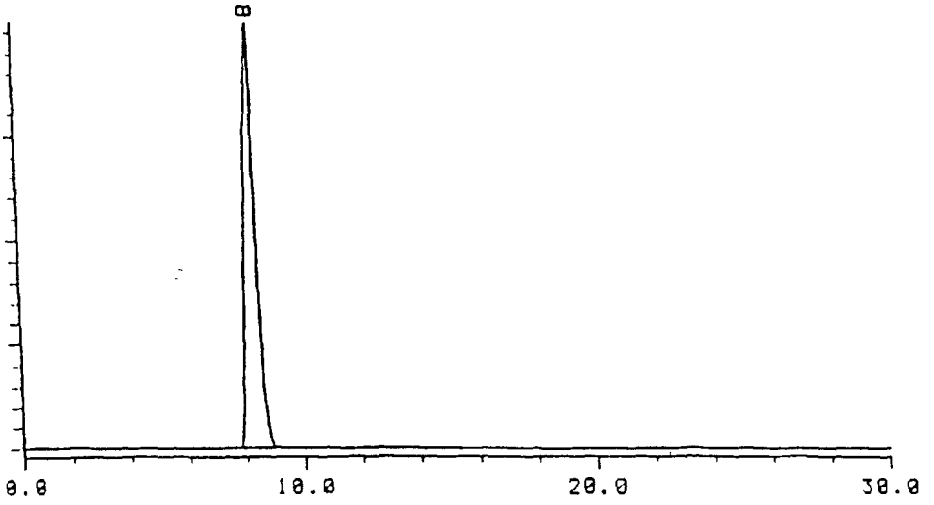
Column: AQ-303 (YMC 4.6 mm ϕ x 250 mm L)
Eluent: Water
Flow rate: 0.5 mllmin
Detector: Differential refractometer
Temperature: Room temperature
Sample Size: 1%, 20 μl

Method: A solution containing 1% (w/v) of the test sample was filtered through a membrane filter (0.45 μm)) to prepare the test solution. The trehalose content of the test sample was calculated from the resultant chromatogram using a simple area ratio method.

Results: Results shown on the following chromatograms verify that the sample contained 100.0% trehalose with no impurities [Figure 7].

Section II. Chemical Identity (Continued)

Figure 7
Hayashibara Trehalose
Liquid Chromatogram Analysis



Retention time (min)

Section II. Chemicalldentitv (Continued)

10. Composition of Elements

Summary: Crystalline dihydrate trehalose was analyzed for carbon and hydrogen using an Automatic Element Analyzer. Results of 37.87% carbon, 6.93% hydrogen and 55.20% oxygen were consistent with theoretical values of 38.10%,6.93% and 54.97%, respectively.

Material: Hayashibara (crystalline dihydrate) Trehalose

Equipment and Apparatus: Automatic Element Analyzer (C, H): MT-type 5 (Yanako Co.) &MT-type3 (Yanako Co.)

Method: This analysis was conducted by Shimazu Techno-Research Inc. (Kyoto, Japan) under, a research contract with Hayashibara Co., Ltd.

Conditions: Test Sample: Crystalline trehalose (Purity 100.0%)

Results: Experimental results were consistent with the chemical formula (C12H22011.2H20) for dihydrate crystalline trehalose shown in Table 5 below.

Table 5

Element	Actual value (%)	Theoretical value[2] (%)
C	37.87	38.10
H	6.93	6.93
O	55.20[1]	54.97

1*calculated figure* 2*calculated as* $C_{12}H_{22}O_{11}.2H_2O$

000094

Section II. Chemicalldentitv (Continued)

11. ← Nuclear Magnetic Resonance Spectrum Analysis (1 H-NMR)

Summary: A resonance, spectrum was produced for Hayashibara trehalose using a nuclear magnetic resonance analyzer with 1H-NMR. Results showed characteristic peaks for pure trehalose.

Material: Hayashibara trehalose

Equipment and Apparatus: High Performance Fourier Transformer Nuclear Magnetic Resonance Analyzer: GSX 400
(Nippon Denshi, Co.)

Method: This analysis was conducted by Shimazu Techno-Research Inc. (Kyoto, Japan) under a research contract with Hayashibara Co., Ltd.

Conditions: TestSample: Crystalline trehalose (Purity 100.0%)

Reference: [2,2,3,3- $^{2}H_{4}$] 3-(trimethylsilyl) sodium propionic acid

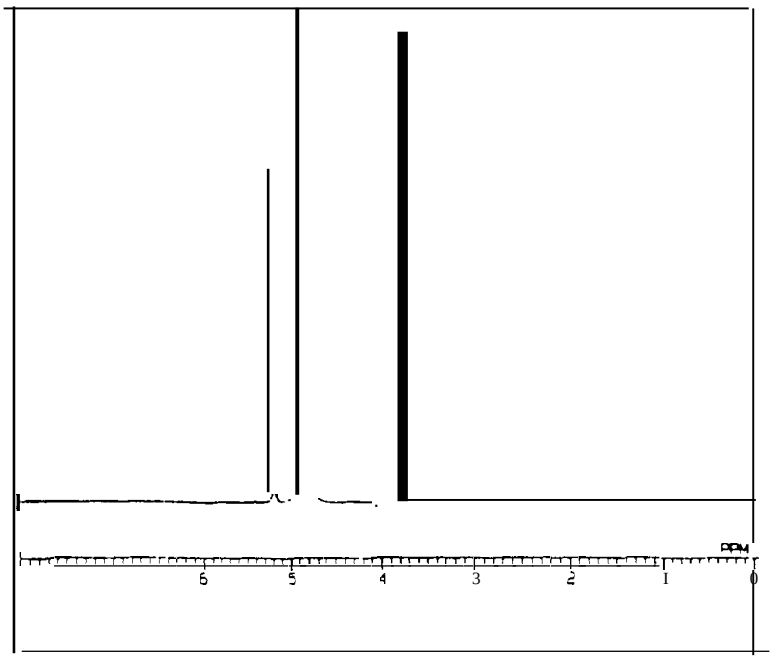
Results: Results shown on the next page [Figure 8] display peak characteristics of pure trehalose: ^{1}H-NMR Spectrum of Hayashibara trehalose (whole spectrum).

Section II Chemical Identity (Continued)

11. Nuclear Magnetic Resonance Spectrum Analysis (1 H-NMR) (continued)

Figure 8
Nuclear Magnetic Resonance Spectrum Analysis (^{1}H-NMR)

Hayashibara Trehalose



Section II. Chemicalldentitv (Continued)

12. Nuclear Magnetic Resonance Spectrum Analysis (13C-NMR)

Summary: A resonance spectrum was produced for Hayashibara trehalose using a nuclear magnetic resonance analyzer with 13C_ NMR. Results showed characteristic peaks for pure trehalose.

Material: Hayashibara trehalose

Equipment and Apparatus: High Performance Fourier Transformer Nuclear Magnetic Resonance Analyzer: GSX 400 (Nippon Denshi, Co.)

Method: This analysis was conducted by Shimazu Techno-Research Inc. (Kyoto, Japan) under a research contract with Hayashibara Co" Ltd.

Conditions:

Test Sample: Crystalline trehalose (Purity 100.0%)

Reference: [2, 2, 3, 3- $^{2}H_{4}$] 3-(trimethylsilyl) sodium propionic acid

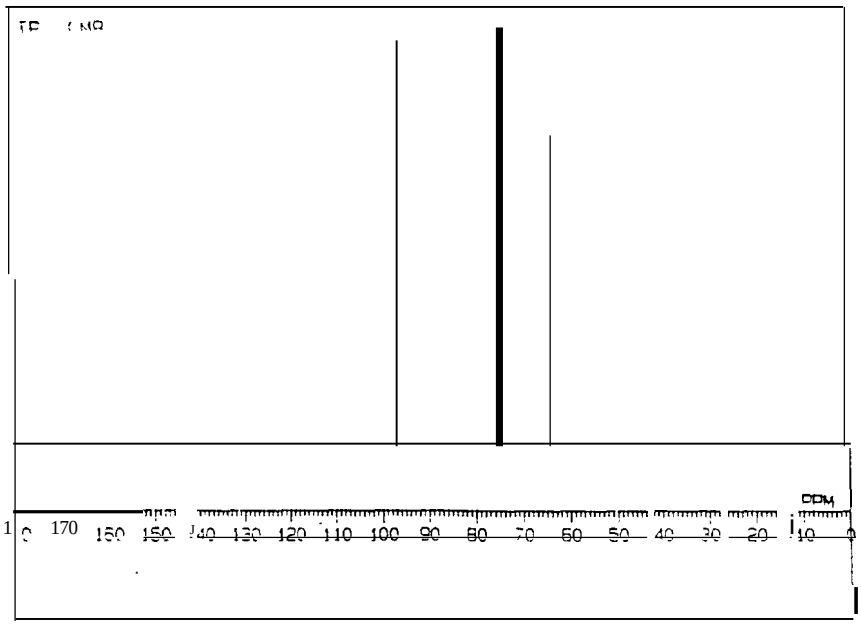
Results: Results shown on the next page [Figure 9] display peak characteristics of pure trehalose: 13C-NMR Spectrum of Hayashibara trehalose (whole spectrum).

Section II. Chemicalldentitv (Continued)

12. Nuclear Magnetic Resonance Spectrum Analysis (13C-NMR) (continued)

Figure 9
Nuclear Resonance Spectrum Analysis
(13C-NMR)

Hayashibara Trehalose



Section II. ChemicalIdentitv (Continued)

I. Comparison of Hayashibara Tretialose with Other Preparations

The melting point and optical rotation of trehalose are two of the most common features that have been reported in the literature. In Table 6 these two variables are compared between Hayashibara trehalose, several other preparations reported in the literature, and other common sugars.

Trehalose has a characteristic melting point and optical *rotation* that allows it to be distinguished from other common sugars. Its has one of the highest positive degrees of optical rotation and does not undergo mutarotation like glucose, fructose and maltose. Comparison of Hayashibara trehalose with other preparations shows that some of the melting points reported overlap those of the other sugars; however, the optical rotations are relatively high and do not change. The reason for the variability or the melting points, as well as the optical rotation, is likely because of remaining contamination in the samples, and *lor* differences in equipment or methods.

Section II. Chemicalldentitv (Continued)

Table 6
Comparison of Hayashibara Trehalose With Other Natural Sources, and to Other Common Sugars

Study	Melting Point	$[a]^{200}$ (water)	Sources
Hayashibara trehalose	97.0°C	+199°	Commercial
Birch (1965)	94-100°C	+180°	Chemical
Wyatt & Kalf (1957)	96.8-97.2°C	+184°	Insects
Lemieux & Bauer (1953) Synthesis	97-98°C		Chemical
Stewart *et al., (1950)*		+179°	Baker's Yeast
Leibowitz (1943)		+198°	Manna
Steiner & Cori (1935)	99-99.5°C	+185°	Yeast
Koch & Koch (1925)	102.5°C	+199°	Yeast
Glucose	83°C	+102°→ +47.9°	
Fructose	103-105°C[1]	-132°→ -92°	
Maltose	102-103°C	+111.7°→ 130.4°	
Sucrose	160-186°C[1]	+66.48°	

'These sugars do not have a melting point, but rather decompose.

000100

Section II. Chemica/Identity (Continued)

J. Hayashibara trehalose Comparison with Naturally Occurring Trehalose

1. Literature Review of Trehalose Extractions from Various Sources

Trehalose exists widely in such diverse organisms as the ergot of rye, lobsters, insects and many common fungi including baker's and brewer's yeast [Birch, 1963, Vol 4 Tab 18]. As such, trehalose is wide spread in nature. Attempts to isolate trehalose from various organisms has been ongoing for several decades, and the trehalose isolated in purified form from such organisms as baker's yeast is usually in the dihydrate crystalline form [Birch, 1965, Vol 19 Tab 4]. The first isolation from baker's yeast was reported by Koch in *Science* in 1925 [Koch and Koch, 1925, Vol 7 Tab 76].

In his 1965 publication, Birch reviewed the physical structure of isolated trehaloses reported in the literature. The melting point and rotations of a,a-trehalose dihydrate were found to range from 96° to 98° and the optical rotation to range from +177° to +197.14°. From this review article, one can draw the conclusion that by approximately 1965, the isolation of trehalose from natural yeast sources had been well described and the yields documented [Birch, 1965, Vol 4 Tab 19].

More recently, researchers have described methods for isolating trehalose from other types of microorganisms. In 1967 Elbein described a method for isolating trehalose from *Streptomycetes,* and in 1969 Suzuki *et al.* published a paper which described the isolation of extracellular trehalose that accumulated in the growth media of *Arthrobacter poraffineus* strain KY 4303 when grown on paraffin as the sale source of carbon [Elbein, 1967, Vol 5 Tab 48 and Suzuki *et al.,* 1969, Vol 12 Tab 130]. The addition of penicillin to the *Arlhrobacter* culture media resulted in a remarkable increase in the accumulation of trehalose, which corresponded to 60-70% of the total sugars accumulated in the culture medium. This finding paved the way for isolation of trehalose from various types of bacteria culture media [Nicolaus *et al.,* 1988, Vol 9 Tab 103].

2. Hayashibara Trehalose

The trehalose produced enzymatically by the Hayashibara Company *is* based on the finding of two novel enzymes isolated from naturally occurring non-pathogenic soil bacteria. The enzymes allow the conversion of starch directly into trehalose

Section II. Chemicalldentitv (Continued)

[Maruta *et al.,* 1995, Vol 8 Tab 89; Nakada *et al.,* 1995a and 1995b, Vol 95 and 96 Tabs 8 and 9]. A third novel enzyme, also discovered in soil bacteria allows the production of trehalose directly from maltose [Nishimoto *et al.,* 1995, Vol 9 Tab 104]. The trehalose produced by the Hayashibara method is equivalent to the trehalose present in human foods such as Mirin, or to that isolated from such diverse organisms as fungi, bacteria and insects [see Table 7] [Aso and Watanabe, 1962a and 1962b, Vol 3 Tabs 2 and 3]. Analysis of a commercial trehalose reveals that the product exhibits a melting point and specific rotation which is consistent with analyse"s reported for trehaloses isolated from naturally occurring organisms by extraction and purification [Elbein, A., 1974 Vol 5 Tab 49]. Therefore, the product of Hayashibara is identical with naturally occurring trehalose.

Section II. Chemical Identity (Continued)

Table 7
Natural Occurrence of Trehalose

Groups	*Specific organisms*
A. Plants	
Pteridophytes (ferns)	*Selaginella* species
	Botrychium iunaria
Spermatophytes(seed plants)	*Echionops persicus*
	Carex brunescens
	Fagus silvalica
B. Algae	
Cynophyceae	*Rivularia bullata*
Rhodophyceae (unicellular)	*Bangia fuscopurpurea*
	Chondrus crispus
	Porphyra laciniata
	Cystoclonium purpurescens
	Furcellaria fastigiata
	Rhodymenia pa/mala
	Lamenea nodosa
	Batr:achospermum
	Se"aticardia marina
C. *Eumycetes* (Fungi and Yeasts)	
Myxomycetes	*Dictyostelium discoideum*
	Dictyostelium mucoroides
Ascomycetes	*Neurospora tetrasperma*
	Claviceps purpurea (ergotof rye)
	Saccharomyces cerevisiae
Basidiomycetes	*Puccinia gaminis*
	Fomes fomentarius
	Fornes annosus

Section II. Chemicalldentitv (Continued)

Groups	*Specific organisms*
C. *Eumycetes* (Fungi and Yeasts) (continued)	
Fungi imperfecti	*Pullularia pullulans*
	Penicillium chrysogenum
	Aspergillus luchuensis
	Myrothecium verracuria
	Pithomyces chartarium
	Sclerotium cepivorum
	Helminthosporium staivum
Mycorrhiza	mycorrhizal roots of beech
D. *Schizomycetes* (Bacteria)	
Actinomycetes	*Mycogbacterium tuberculosis*
	Other mycobacteria
	Nocardia species
	Streptomyces species
Other bacteria	*Corynebacteria* species
E. Anthropods (Insects)	
Orthoptera	
House cricket	*Gryllus domesticus*
Cockroach	*Leucophaea maderae*
Grasshopper	*Melanophus differentialis*
Cockroach	*Perplantea americana*
Locust	*Schistocerca gregaria*
Homoptera	
Aphid	*Megoura viciae*
Lepidoptera	
Silkworm	*Antheraea pernyi*
	Antheraea polyphemus
	Bombyx mori
Hawk Moth	*Celerio euphorbiae*
	Deilephila elpenor
Wax moth	*Glleria mellonella*
	Hya/ophora ceropia
Armyworm	*Leucania* spearata
Eri (silkworm)	*Samia cynthia*
	Sphinx ligustri

Section II. Chemicalldentitv (Continued)

Groups	*Specific organisms*
E. Anthropods (Insects (continued)	
Tobacco worm	*Protoperce* sexta
Silk Moth	*Telea polyphemus*
	Playsamia cecropia
Coleoptera	*Chalcophora mariana*
Water beetle	*Dyliscus marginalis*
	Hydrous piceus
Meal worm	*Tenebrio molitor*
Flour beetle	*Tribolium confusum*
Hymenoptera	*Anthophora* sp.
Honeybee	*Apis mellifera*
Sawfly	Diprion hercyniae
	Trichiocampus populi
Diptera	
Horse botfly	*Gastrophilus intestinalis*
Blowfly	*Phormia regina*
Mosquito	*Aedes aegypti*
Fruit fly	*Drosophila repleta*
Hemiptera	
Milkweed bug	*Oncopellus fasciatus*
F. Other Invertebrates	
Parastic worm	*Ascaris lumbricoides*
Lobster	*Homarus americanus*
	Carcinus macenus
Shrimp	*Artemia salina*
Nematode	*Nippostrongylusbrasiliensis*
Helminth	*Moniliformis dubius*
Annelid	
Sandworm	*Diopatra cupreae*
	Glycera dibranchiata
Earthworm	*Lumbricus terrestus*
Leech	*Dina fervida*
	Glassosiphonia complanata
	Hellobdella stagnalis
Peanut worm	*Golfingia gouldii*

Elbein, A., 1974. *Advances in Carbohydrate Chemistry.* 30:229, [Vol 5 Tab 49].

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Section II. Chemicalldentitv (Continued)

K. Physicochemical Properties of Trehalose Which Form the Basis for its Potential Functionality in Food

1. Introduction

Trehalose is a unique non-reducing conformationally stable disaccharide which has been described as both necessary and sufficient for the protection of various cryoptobiotic and anhydrobiotic organisms during periods of freezing or desiccation [Colaço *et al.,* 1994, Vol 4 Tab 34]. Through convergent evolution such diverse organisms as brine shrimp and baker's yeast have developed the ability to accumulate trehalose during times of stress [Van Dijck *et al.,* 1995, Vol 12 Tab 136). For example, baker's yeast can accumulate trehalose to levels between 15 and 20% of dry weight [Colaço *et a/.,* 1992, Vol 4 Tab 33). The repeated independent selection of trehalose as a protectant by taxonomically diverse organisms is an important clue from nature about its value. Recently, trehalose has been used in the biopreservation of bacteria, antibodies and delicate enzyme systems which were dried using a variety of processes [Leslie *et al.,* 1995, Vol 7 Tab 82 and Colaço *et al.,* 1992 Vol·4Tab 36]. Because of its unique properties, which include a slight sweetness, high solubility, chemical non-reactivity, low hygroscopicity, stability, and lack of color and odor, trehalose has been suggested as a potential ingredient to protect foods against damage caused by drying freezing or destructive chemical reactions [Miyake, 1997, Vol 8 Tab 94; Birch, 1970, Vol 4 Tab 20; Roser, 1991a, Vol 9 Tab 113; Colaço, 1994, Vol 4 Tab 34; Roser, 1993, Vol 9 Tab 115, Roser, 1991b, Vol 9 Tab 114; Leslie *et al.,* 1995, Vol 7 Tab 82; and Colaço *et al.,* 1992, Vol4Tab 36). While the protecting mechanism of trehalose has not been clearly understood several hypotheses have been the subjects of active research.

2.. Trehalose Mechanism of Action

The mechanisms proposed for the protective effects of trehalose are summarized below:

a. Trehalose may protect delicate cell membranes by altering the chemistry of water [Donnamaria *et a/.,* 1994, Vol 5 Tab 46].

Section II. Chemicalldentitv (Continued)

b. The "water replacement hypothesis" is based on the ability of trehalose to substitute for structural water by forming

c. hydrogen bonds with macromolecular structures such as proteins [Roser, 1991a, Vol 9 Tab 114, and Kawai *et al.,* 1992, Vol 7 Tab 74].

d. Trehalose forms'an amorphous glassy solid state during freezing and drying may suspend the vital tertiary structures of proteins and phospholipids in a stable matrix [Cola90 *et al.,* 1994, Vol 4 Tab 34, Crowe *et al.,* 1990, Vol 5 Tab 39; and Rudolph and Crowe, 1985, Vol 9 Tab 116].

e. Several investigators have suggested that it is the unique chemical inertness of trehalose that may be important in its membrane protective properties [Cola90 *et al.,* 1994, Vol 4 Tab 34].

3. Potential Uses of Trehalose in Food Preservation

Several potential uses for trehalose have been cited in the literature. Roser, 1991 :has advocated the use of air drying with trehalose as a cryoprotectant to replace expensive freeze-drying of foods and pharmaceuticals. The author cites the fact that even freeze-dried products have a limited shelf-life due to the damage done to proteins during desiccation [Roser, 1991a, Vol 9 Tab 113].

The UK has recently granted trehalose approval as a cryoprotectant [Tab 14], and the universality of its effectiveness has been recounted by Roser in a publication in which he stated that his laboratory had yet to find a biological molecule, molecular complex or mixture of biomolecules that could not be successfully dried in trehalose-containing buffers, stored at high temperatures and then rehydrated with a recovery of close to 100% of the original biological activity [Roser, 1991a, Vol 9 Tab 113].

The only constraint placed on the simplicity of this procedure was that any molecule air dried with trehalose must be in a buffer containing all the components required for its active configuration. For example the ionic composition of the buffer needed to be appropriate for the particular material being dried. If the magnesium requiring enzyme alkaline phosphatase was dried in buffers lacking divalent magnesium for instance, the activity would

Section II. Chemicalldentitv (Continued)

be blocked even when trehalose was present. In Roser's laboratory the enzyme was found to be fully active when trehalose-dried in the presence of the correct concentration of magnesium [Roser, 1991, Vol 9 Tab 113). The author concluded that because metal ions are an essential structural element of these metalloproteins it was not surprising that they are required during drying.

Bolin reported in 1987 that fruits which are cut and dried required some type of treatment if they were to retain their light color [Bolin and Steele, 1987, Vol 4 Tab 25]. These types of treatment have often consisted of sulfites or sulfur dioxide. The object of a study reported by Bolin was to screen for other routes for the prevention of browning in fruits because sulfites were known to cause allergic reactions in some individuals. Bolin reported that packing of dried apples for instance, with oxygen scavengers markedly decreased product darkening during storage, and that about 20% of the non-enzymatic browning reactions occurring during storage could be attributed to Maillard reactions, while approximately 70% were due to oxidation reactions. Maillard reactions are actually a cascade of chemical reactions initiated by the spontaneous condensation of reactive carbonyl and amino groups such as those commonly occurring between reducing sugars and proteins [Karmas and Karel, 1992, Vol 7 Tab 37 and Bolin and Steele, 1987, Vol4 Tab 25]. Bolin reported that trehalose was one substance that inhibited browning reactions and could be used as a substitute for sulfite [Bolin, and Steele, 1987, Vol 4 Tab 25].

Karmas *et al.,* also demonstrated the ability of trehalose to protect proteins from Maillard reactions [Karmas and Karel, 1992, Vol 7 Tab 37]. Their system included xylose and lysine because of their high reactivity. Browning results for solutions consisting of matrixlxylosellysine (94:5:1) at 20·C showed that matrixes containing trehalose resisted browning twice as long (4 weeks versus 2 weeks) as those containing lactose or raffinose. One solution containing maltodextrin and trehalose (47:47) retarded browning at water activities ($A_{w)}$ ranging from 0.0 to 0.9. The authors indicated that this results was due to the inhibition of crystallization. Normally crystallization and hence browning is initiated at the A_w where the glass transition temperature drops below the storage temperature, in this case (20°C).

Section II. Chemicalldentitv (Continued)

McDonald and Lanier discussed trehalose as a cryoprotectant for meat and surimi [MacDonald *et al.,* 1991, Vol 8 Tab 84]. Banana slices, soaked in a 10% *w/w* trehalose solution before drying retained the color and texture of fresh banana, as did fresh herbs [Colaço *et al.,* 1994, Vol 4 Tab 34]. The colors of the fresh food stuffs were reported to be preserved in the dried products even on extended storage and when rehydrated, the characteristic flavor and aroma of the fresh products were regained [Colaço *et a/., 1994,* Tab 6]. Colaço *et a/.,* reported that food volatiles were preserved, such as in mangos and bananas, and that there was a significant decrease in the amount of the degradation compounds furfural and a-humulene produced in the trehalose dried samples [Coloço *et al.,* 1994, Vol 4 Tab 34].

4. Conclusion

The ubiquitous selection of trehalose as a preservative in nature has been well documented. Its use in a wide variety of foods in Japan demonstrates its value in stabilizing the texture, structure, color, and water content of food products. Further development in the applications area will undoubtedly broaden the field of use for this sugar.

L. Physicochemical Properties

Section S contains test results on the various physicochemical properties of Hayashibara trehalose, which are likely to have a bearing on its behavior in food systems.

1. Hvaroscopicitv

Summary :_Samples of both the anhydrous and dihydrate forms of trehalose were placed in desiccators with relative humidities ranging from 33.0 to 97.3% and stored for 7 days. Results showed that the dihydrate form was hygroscopic, increasing from 9.54% to approximately 18% moisture only at 97.3% relative humidity. The relatively non-anhydrous form increased steadily in moisture content as humidity rose.

Material: Hayashibara trehalose (dihydrate crystalline and anhydrous crystals)

Section II. Chemical Identity (Continued)

Method: One (1) g of the. crystalline sample was placed in a desiccator containing salt solution and a known relative humidity. The test sample was stored at 25°C for 7 days, after which time, the sample was weighed 3 times. The mean of the three values was recorded as the water content. Anhydrous trehalose was tested for changes in weight on days 1, 3 and 7.

Conditions: Test sample: Hayashibara trehalose (Purity: 100%)
Water content of the test samples: Dihydrate trehalose 9.54%
Anhydrous trehalose: 0.65%

Desiccator with controlled humidity: Saturated solutions known to produce specific relative humidities at 25°C were placed in the desiccators.

RH(%)	Saturated solutions	
33.0	magnesium chloride	[MgCI2-6H20]
52.8	magnesium nitrate	[Mg(N03)2-6H20]
60.0	ammonium nitrate	[NH4N03]
75.2	sodium chloride	[NaCl]
84.2	potassium chloride	[KCl]
90.1	barium chloride	[BaCI2-2H20]
92.4	potassium nitrate	[KN03]
97.3	Potassium sulfate	[K2S04]

Results: Results showed that the dihydrate form remained stable in moisture content up to approximately 92.4% RH. The moisture content of the anhydrous form paralleled the increase in humidity and rose from 0.65% to nearly 14% at 97.3% RH.

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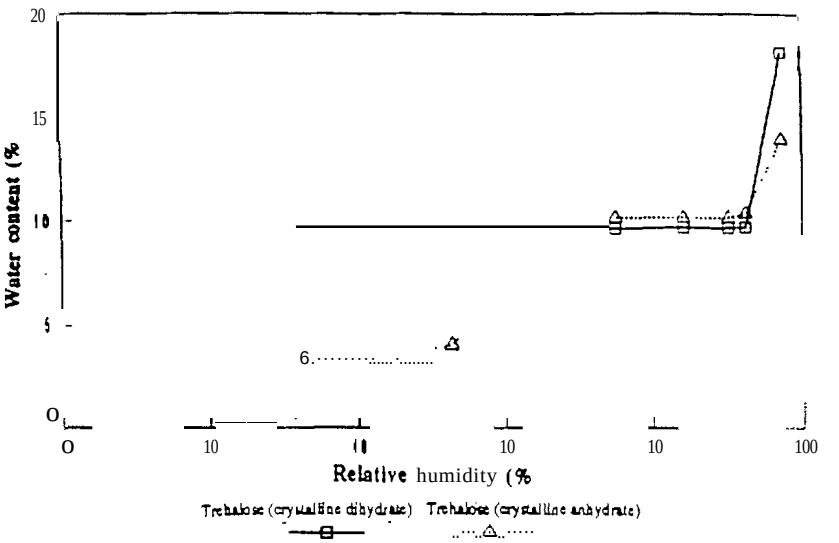
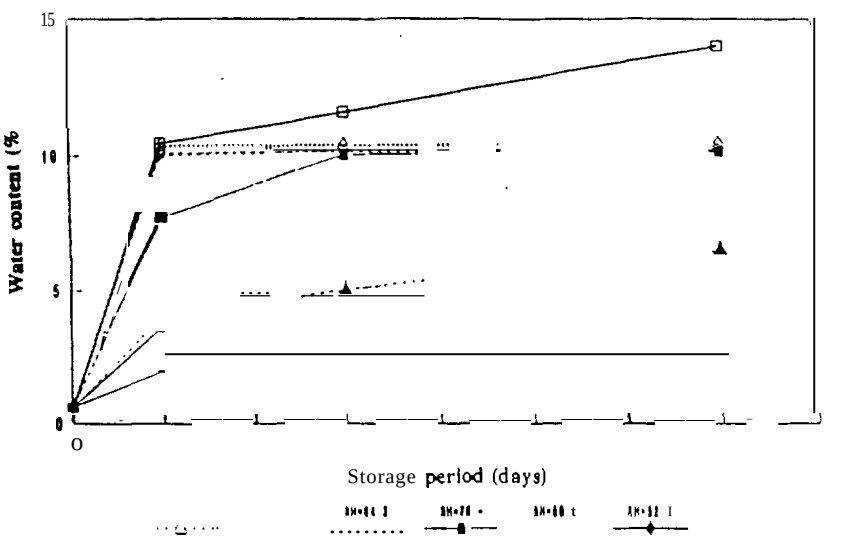
Section II. Chemical Identity (Continued)

Figure 10
Water content of dihydrate crystalline trehalose at different relative humidities after 7 days.



Watel' content of anhydrous cl'ystalline h'ehnlnse at diffel'ent relative humiclilies after 7 days.



Section II. Chemicalldentitv (Continued)

2. Water Solubility

Summary: The solubility of Hayashibara's trehalose was measured at various temperatures ranging from 10-90°C. The test sample was added to deionized water in quantities 5-10% greater than the amount expected to dissolve. The sugar concentration was calculated by decompression. Solubilities ranged from 35.6% and 85.5% and were directly related to temperature.

Material: Hayashibara trehalose (dihydrate crystals)

Method: Varying weights (5-10% more than the amount expected to solubilize at each temperature) of the test sample and 30 grams deionized water were placed in a 100-ml flask and sealed. The flask was rotated slowly in a thermo bath at each temperature. The sugar concentration of the solutions was calculated by decompression, heat and dry method (diatomaceous method). Microcrystals were removed from each solution by glass fiber filtration paper. The water solubility was obtained by periodic sampling. The point at which the sugar concentration of the solution reached equilibrium was recorded as the saturation point.

Conditions:
Test Sample: Hayashibara trehalose (Purity 100%)
Temperatures: 10, 20, 30, 40, 50, 60, 70, 80, 90°C

Results: The solubility data summarized below demonstrated that the solubility of trehalose increased with temperature and ranged from 35.6% at 10°C to 85.8% at 90°C.

Table 8
Water Solubility of Hayashibara Trehalose (Anhydrous Basis)

Temp. (°C)	10	20	30	40	50	60	70	80	90
g/100g water	55.3	68.9	86.3	109.1	140.1	184.1	251.4	365.9	602.9
Saturation concentration (%, w/w)	35.6	40.8	46.3	52.2	58.3	64.8	71.5	78.5	85.8

Section II. Chemical Identity (Continued)

3. Osmotic Pressure

Summary: An osmometer was used to measure the osmotic pressure of solutions containing varying concentrations of either trehalose or maltose. Results showed that the osmotic pressures of the two sugars were nearly identica.l given equal concentrations.

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus: Osmometer: OSM-type 1 (Shimazu Seisakusho Co.)

Method: Solutions with different concentrations of the test sample were tested by a vapor pressure method using an Osmometer at 37°C. Maltose was used as the control.

Conditions:
Test Sample: Hayashibara trehalose (Purity 100.0%)
Control (maltose) solution: Maltose (Purity: 99.2%)
(Hayashibara Biochemical Labs., Inc.)
Concentration of solutions: 5,10,20,30% *(w/w)*

Results: The results summarized below demonstrated that the osmotic pressure ofvarying concentrations of either maltose or trehalose were nearly identical.

	Concentration (%, *w/w)*			
Osmotic pressure (mOsm)	5	10	20	30
Trehalose	193	298	690	1229
Maltose	195	299	676	1221

Section II. Chemical Identity (Continued)

4. pH Stability

Summary: Solutions containing 4% trehalose were buffered with 0.02 mM of various pH buffers. These test solutions were heated to 100°C for 0,8 and 24 hours. pH measurements were taken at each sampling period. Results showed that the trehalose solutions were pH stable for up to 24 hours. Residual sugar content as measured by HPLC- remained virtually unchanged.

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus: pH meter: HM-60S (Toa Denpa Kogyo Co.)
HPLC: Pump - CPD (Toso Co.)
Detector: differential refractometer (Toso Co.)

Method: Solutionscontairiing 4% test sample and 0.02 M of each pH buffer solution were sealed in test tubes with screw caps and were then treated by heating at 100°C in an oil bath. After cooling, the test solutions were analyzed for pH and the residual ratio of the test material. The residual ratio was calculated from compositions determined by HPLC after deionization of the test solution.

Conditions: Test Sample: Hayashibara trehalose (Purity 100%)

Buffer solutions:
pH 2-5 (acetate buffer solution)
pH 6-8 (phosphate buffer solution)
pH 9-10 (ammonium buffer solution)

Heating temperature: -100°C
Heating times: 0, 8. 24 hours

Deionization Ion exchange resins:
IRA-411 (ion-): SK 1B (ion+) = 2:1

HPLC conditions,:
Column: PA-03 (YMC)
Solvent: acetonitrile: water = 70:30 (v/v)
Flow rate: 0.75 ml/min.

Results: Hayashibara trehalose was stable in solution between pH 3.5 and pH 10.0 for 24 hours, and the residual sugar ratio remained

Section II. Chemical Identity (Continued)

stable after treatment with various pH buffers. Data from the pH stability study is summarized below.

	pH of test solution after treatment with different pH buffers		
2	3.46	3.41	3.83
3	3.71	3.64	4.02
4	4.25	4.22	4.50
5	5.14	5.10	5.25
6	6.69	6.67	6.52
7	7.46	7.44	7.13
8	8.25	8.14	7.64
9	8.88	8.91	8.42
10	9.86	9.85	9.16

Residual ratio (% trehalose) after treatment with different pH buffers

	Treatment time (hr)		
pH	0	8	24
2	100.0	100.0	100.0
3	100.0	100.0	99.2
4	100.0	99.7	100.0
5	100.0	99.5	100.00
6	100.0	100.0	99.5
7	100.0	99.5	100.0
8	100.0	99.5	99.7
9	100.0	100.0	99.7
10	100.0	100.0	99.8

5. Heat Stability

Summary: A 10% solution of trehalose buffered to pH 6.0 was heated at 120°C for up to 90 minutes. Results of color analysis as measured at 480 nm with a spectrometer showed that heat had no effect on the trehalose solution, while a 10% maltose solution used as control increased in color in direct proportion to the length of the heating period.

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Section II. Chemicalldentitv (Continued)

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus: Spectrophotometer: UV-240 (Shimazu Seisakusho Co.)

Method: Solutions containing a 10% test sample with 0.033 M phosphate buffer solution (pH 6.0) were sealed in test tubes with screw caps and treated by heating at 120°C in an oil bath. After cooling, the test solutions were analyzed for absorbance at 480 nm using a 1-cm cell layer. The absorbance was designated as the color value. Maltose was used as the control.

Conditions:
Test Sample: Hayashibara trehalose (Purity: 100%)
Maltose: Purity 99.2% (Hayashibara Biochemical Labs.)
Heating times: 0, 30, 60, 90 min.

Results: The results summarized below showed that the solution of 10% trehalose was not affected by heating, while the color value of a 10% maltose solution increased with longer heating periods.

Treatment time (min)	Color value	
	Trehalose	Maltose
0	0.005	0.000
30	0.013	0.051
60	0.010	0.121
90	0.012	0.184

6. Heat Stability (With Glycine)

Summary: Solutions containing 1% glycine and either 10% maltose or 10% trehalose were heated to boiling in the presence of 0.05 M buffer solutions which were used to adjust the pH levels of the mixed solutions. Results showed that color value as measured with a spectrometer remained stable over a range of pH levels (4.0-7.0) for the trehalose containing solutions, while the solution containing

Section II. Chemical Identity (Continued)

maltose increased in color steadily with increasing pH levels and with increasing exposure to heat.

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus: Spectrophotometer: UV-240 (Shimazu Seisakusho Co.)

Method: Solutions containing a 10% trehalose 1% glycine prepared with a 0.05 M buffer were sealed in test tubes with a bead on the top of the tube.. The test solutions were heat treated in boiling water for various lengths of time from 0 - 90 minutes. After cooling, the test solutions were analyzed for absorbance at 480 nm using a 1-cm cell layer. The absorbance was designated as the color value. Maltose was used as the control.

Conditions: Test sample: Hayashibara trehalose (Purity: 100%)
Maltose: Purity 99.2% (Hayashibara Biochemical Labs.)
Glycine: Special reagent grade (Wako Jyunyaku Co.)
Buffer solutions: pH 4.0, 5.0 (acetic acid buffer)
pH 6.0, 6.5, 7.0 (phosphate buffer solution)
Heating times: 0, 30, 60, 90 minutes

Results: The results summarized below demonstrated that a 10% trehalose solution in combination with 1% glycine was stable with regard to color when heated to boiling at various pH levels. The color stability was unaffected by the length of heating time. A control solution containing maltose and glycine increased in color with both time and pH.

Section II. Chemicalldentitv (Continued)

Table 9
Heat Stability of Hayashibara Trehalose with Glycine

Treatment time (min)		Color value pH4.0	pH 5.0	pH 6.0	pH 6.5	pH 7.0
0	Trehalose	0.000	0.000	0.000	0.000	0.000
	Maltose	0.000	0.000	0.000	0.000	0.000
30	Trehalose	0.000	0.001	0.006	0.006	0.003
	Maltose	0.000	0.001	0.039	0.085	0.128
60	Trehalose	0.000	0.001	0.010	0.022	0.018
	Maltose	0.002	0.020	0.169	0.319	0.486
90	Trehalose	0.000	0.002	0.010	0.010	0.012
	Maltose	0.003	0.014	0.324	0.610	0.926

7. Heat Stability (With Proteins)

A 10% *(w/w)* solution of trehalose was combined with a 5% *(w/w)* solution of polypeptone as a protein source. Sealed tubes were heated to 120°C and held at temperature for varying lengths of time. After cooling the solutions were diluted to an appropriate concentration and analyzed for absorbency at 480 nm. The color value of the trehalose solutions was compared to controls containing maltose.

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus: Spectrophotometer: UV-240 (Shimazu Seisakusho- Co.)

Method: A solution containing 10% *(w/w)* of the test sample was combined with a 5% *(w/w)* solution, of polypeptone and sealed in a test tube with a screw cap. MUltiple tubes were treated by heating

Section II. Chemicalldentitv (Continued)

at 120°C in an oil bath. After cooling, the test solutions were diluted to an appropriate test range and ana_lyzed for absorbance at 480 nm using a 1-cm cell layer. The absorbance multiplied by the dilution factor was designated as the color value. Maltose (10%) was used as the control. The trehalose value obtained with a solution containing polypeptone alone (as a blank) was deducted from the value above.

Conditions:
Test Sample: Hayashibara trehalose (Purity: 100%)
Maltose: Purity 99.2% (Hayashibara Biochemical Labs.)
Polypeptone: For bacteria. culture media (Dainippon Seiyaku Co.)
Heating times: 0, 30, 60, 90 min.

Results: The results summarized below demonstrated that the change in color value in the trehalose solutions was insignificant, while those for maltose solutions increased with time.

Table 10
Changes in the Color Value of Trehalose Solutions heated in the Presence of Protein

Treatment time (min)	Color value	
	Trehalose	Maltose
0	0.007	0.005
30	0.011	0.483
60	0.008	2.412
90	0.014	5.019

8. Storage Stability of Solution

In order to establish the storage stability of a 10% solution of trehalose, aliquots of the solution *Were* sealed in glass tubes and stored at 25 and 37°C in the dark. Samples were tested for pH, color, turbidity and residual sugar ratio, over a 12 month period. Results were reported every 30 days for the first 6 months, and then at months 9 and 12.

Section II. Chemicalldentitv (Continued)

Material: Hayashibara trehalose (dihydrate crystals)

Equipment and Apparatus:
pH meter: HM-60S (Toa Denpa Kogyo Co.)
Spectrophotometer: UV-160 (Shimazu Seisakusho Co.)
HPLC: Pump - CCPD (Toso Co.)
Detector: differential refractometer (Toso Co.)

Method: Eight (8)-ml aliquots of a solution containing 10% *(w/v)* test sample were sealed aseptically in 10 ml test tubes with screw caps. The test tubes were stored at 25 and 37°C in a dark place. The stored solutions were periodically tested for pH, color value, turbidity and the residual ratio of the test material.

Conditions:
Test Sample: Hayashibara trehalose (Purity: 100%)
Storage temperatures: 25,37°C
Storage period: 0, 1,2, 3,4, 5,6,9, 12 months
Colorization: Absorbance at 480 nm (1 cm)
Turbidity: Absorbance at 720 nm'(1 cm)
Residual ratio of test sugar: HPLC: solutions mixed with equal amounts of glycine (same concentrations as the respective stored sugar solutions) and the test solutions.
Glycine: Reagent (special grade) (Wako Jyunyaku Co.)
HPLC conditions: Column: PA-03 (YMC)
Solvent: acetonitrile: water =73:27 (v/v)
Flow rate: 0.8 ml/min.

Results: . The study showed that virtually no change in color, turbidity and residual sugar content occurred during the test. pH values dropped from 6.80 to 5.27 at 25°C and from 6.80 to 5.15 at 37°C over the course of the study. The conclusion of the study was that trehalose solution was stable over a 12 month storage period.

9. Degree of Sweetness

A taste panel was asked to compare the sweetness of a 22.2% trehalose solution with various sucrose solutions ranging from 8-*12% (w/w).*

Section II. Chemicalldentitv (Continued)

Material: Hayashibara trehalose (dihydrate crystals)

Method: A closed taste panel test was conducted with 10 volunteers using a 22.2% *(w/w)* test solution of trehalose and control solutions containing from 8-12% sucrose. Each volunteer drank one each of 5 sucrose control solutions from lower to higher concentration and then from higher to lower concentration (total 10 solutions tested). The purpose of this design was to avoid the influence of concentration in the panelist's response. The sweetness of the test solution was calculated based on the concentration of the control solution which most closely corresponded to each test solution, and on the sweetness of the control solution equaling 100.

Conditions:
Test Sample: Hayashibara trehalose (Purity 100%)
Control solution: Sucrose (granulated sugar) (Daichi Sugar Co.)
Concentration of control solution: 8. 9, 10, 11, 12% *(w/w)*
Temperature of solution: Room Temperature
Testers: 10 females, (20 responses because each tester performed the test twice)
Responses: 20 (each tester tasted solutions in duplicate)

Results: Results showed that the trehalose solution was about 45% as sweet as a corresponding 10% sucrose solution.

Test solution	Trehalose (22.2%) solution				
Control solution (% sucrose)	8%	9%	10%	11%	12%
Sweetness	36	41	45	50	54
No. of responders who responded:					
test solution is sweeter than control	20	15	3	O	O
same	O	5	8	2	O
control is sweeter than test solution	O	O	9	18	20

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Section II. Chemical Identity (Continued)

10. Quality of Sweetness

A taste panel rated the quality of sweetness of a 22.2% trehalose solution and a 10% sucrose solution. Each of 10 testers responded in duplicate.

Material: Hayashibara trehalose (dihydrate crystalline)

Method: A closed taste panel was conducted with 10 testers using a 22.2% (w/w) test solution and control solutions. Each tester was requested to drink one each of 2 control solutions from lower to higher concentration and from higher to lower concentration to avoid any concentration influence of the control solutions. A taste preference test method was used. Judgment was made based on a two dimensional preference rating chart (risk ratio: 5%). When either one of the solutions was selected by more than 10 of 20 responders, it was judged as a significant difference between the two.

Conditions:
Test sample: Hayashibara trehalose (Purity: 100%)
Control so.lution: Sucrose (granulated sugar) (Daiichi Sugar Co.)
Concentration of control solution: 5, 10% (w/w)
Temperature of solution: Room temperature
Panel Testers: 10 females, (20 responses because each tester performed the test twice)

Results: Results showed that 17 of 20 responses recorded a preference for trehalose over when a 22.2% trehalose solution was compared with a 10% sucrose solution.

Test solution:	Trehalose (22.2%) solution
Control solution:	Granulated sugar 10%
No. of responses who responded:	
Test solution is superior	17
Control solution is superior	3

000122

Section II. Chemicalldentitv (Continued)

11. Viscosity of Solution

The viscosity of various test solutions of trehalose was measured with a viscometer at 25 and 37°C. The solutions ranged in concentration from to' 40% *(wlw).*

Material: Hayashibara trehalose (crystalline dihydrate)

Equipment and Apparatus: Viscometer: B type viscometer Tokyo Keiki Co.)

Method: Solution samples containing different concentrations of the test sample were prepared and the viscosity was measured at 25 and 37°C by viscometry.

Conditions:
Test Sample: Hayashibara trehalose (Purity: 100%)
Concentration of test solution: 0, 5, 10,20, 25, 30, 35, 40% *(w/w)* [determined by decompression, heat and dry method (diatomaceous method)].
Temperatures: 25°C, 37°C

Results: Results showed.that even at relatively high concentrations, the viscosity of trehalose solutions remained relatively low «6.0cP). Increasing concentrations of trehalose in solution had little effect on viscosity.

Hayashibara Trehalose Viscosity at Various Concentrations

Concentration (%,wlw)		0	5	10	15	20	25	30	35	40
Viscosity(cP)	25°C	0.96	1.10	1.21	1.47	1.78	2.11	2.95	4.02	5.65
	37°C	0.82	0.83	0.96	1.14	1.37	1.73	2.21	3.02	4.03

M. Potential Human Toxicants

As described above, Hayashibara trehalose is produced using a crystallization process. This type of processing assures that potential contaminants (such as lead), which would be of concern for human health are not present in the final product. An analysis of five lots in duplicate has shown that there is no evidence that Hayashibara trehalose is a source of significant contamination. The purity of the product has been

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Section II. Chemical Identity (Continued)

demonstrated with analytical results, and long-term shelf studies (See Section II).

N. Specifications for Food Grade Materials

1. Raw Materials

The following substances used in the manufacturing of trehalose are food grade materials, or are used in accordance with current Good Manufacturing Practices (21 CFR 110.80(a)).

Activated Carbon
Alpha-amylase (EC 3.2.1.1)
Calcium Carbonate
Calcium Chloride
Corn Starch
Cyclomaltodextrin Glucano-transferase (CGTase; EC 2.4.1.19)
Diatomaceous Earth
Glucoamylase (EC 3.2.1.3)
Hydrochloric Acid
Ion Exchange Resin
Isoamylase (EC 3.2.1.3)
Parlite
Sodium Chloride
Sodium Hydroxide
Thermostable alpha-amylase (EC 3.2.1.1)
Trehalose Producing Enzyme
    Maltooligosyl-trehalose synthase (EC 5.4.99.15)
    Maltooligosyl-trehalose trehalohydrolase (EC 3.2.1.141)

2. Specifications of Raw Materials Set by Hayashibara
(See also Table 1)

Activated Carbon

Variables	Specifications
Chloride (as Cl)	≤ 0.53%
Sulfate (as S04)	≤ 0.48%
Zinc (as Zn)	≤ 0.10% μg/g
Lead (as Pb)	≤ 10 μg/g
Arsenic (AS203)	≤ 4.0 μg/g

Section II. Chemicalldentitv (Continued)

N. Specifications for Food Grade Materials (continued)

Calcium Carbonate

Variables	Specifications
Content	98.0-102.2% ($CaCO_3$) after drying
Hydrochloric Acid Insoluble Substances	≤ 0.20%
Heavy Metals (as Pb)	≤ 20 μg/g
Alkali Metals and Magnesium	≤ 1.0%
Barium (as Ba)	≤ 0.030%
Loss on Drying	≤ 2.0%

Calcium Chloride

Variables	Specifications
Appearance	slightly turbid
Content	≤ 70.0% ($CaCl_2$)
Heavy Metal (as Pb)	≤ 20 μg/g
Alkali Metals and Magnesium	≤ 5.0%
Arsenic (AS203)	≤ 4.0 μg/g

Corn Starch

Variables	Specifications
Water Content	≤ 13.5%
pH	4.0-5.0
Starch content	≥ 95%
Crude proteins	≤ 0.35%

Section II. Chemicalldentitv (Continued)

N. Specifications for Food Grade Materials (continued)

Cyclomaltodextrin Glucano-transferase (CGTase)

Variables	Specifications
Heavy Metals (as Pb)	≤ 10 ppm
Arsenic	≤ 1 ppm
pH	6.5-8.0

Diatomaceous Earth

Variables	Specifications
pH	5.0-10.0 (dry or barned product) 8.0-11.0 (solvent barned product)
Water Soluble Substances	≤ 0.50%
Hydrochloric Acid Soluble Substances	≤ 2.5%
Heavy Metal (as Pb)	≤ 50 μg/g
Lead (as Pb)	≤ 10 μg/g
Arsenic (AS203)	≤ 10 μg/g

Glucoamylase

Variables	Specifications
Heavy Metals (as Pb)	≤ 40 ppm
Arsenic	≤ 3 ppm

Section II. Chemicalldentitv (Continued)

N. Specifications for Food Grade Materials (continued)

Hydrochloric Acid

Variables	Specifications
Content	90-120% of the labeled value
Sulfate (as S04)	*≤ 0.48%(w/v)*
Heavy Metals (as Pb)	≤ 10 μg/ml
Iron (as Fe)	≤ 30 μg/ml
Arsenic as(AS203)	≤ 2.0 μg/ml
Residue on Ignition	≤ 0.020%

Ion Exchange Resin

Variables	Specifications
Solid Content	≤ 25%
Water Soluble Substances	≤ 0.50%
Heavy Metals (as Pb)	≤ 20 μg/g
Arsenic (AS_2O_3)	≤ 10 μg/g
Total Ion Exchange Capacity	ion + resin:
	ion - resin'

Isoamylase

Variables	Specifications
Heavy Metals (as Pb)	≤ 10 ppm
Arsenic	≤ 1 ppm
pH	6.5-8.0

000127

Section II. Chemicalldentitv (Continued)

N. Specifications for Food Grade Materials (continued)

Thermostable alpha-amylase

Variables	Specifications
Calcium Carbonate	30-40%
Heavy Metals (as Pb)	≤ 10 ppm
Arsenic	≤ 1 ppm

Alpha-amylase

Variables	Specifications
Heavy metals	≤ 10 ppm
Arsenic	≤ 1 ppm

Parlite

Variables	Specifications
pH	5.0-9.0
Water Soluble Substances	≤ 0.20%
Hydrochloric Acid Soluble Substances	≤ 2.5%
Heavy Metals (as Pb)	≤ 50 μg/g
Lead (as Pb)	≤ 10 μg/g
Arsenic (AS203)	≤ 4.0 μg/g
Residue on Ignition	≤ 3.0%
Hydrogen Fluoride Residues'	≤ 37.5%

Notification Section III

000129

Section III. Information on Self-Limiting Levels of Use and Probable Consumption

A. Self-Limiting Levels of Use

Hayashibara trehalose is believed to have a variety of functional uses that will make it a valuable ingredient in a large number of applications. However, in calculating estimated daily intake levels, Hayashibara submits that there are several factors that will effectively limit the use of trehalose in foods.

1. Trehalose is relatively more expensive than other carbohydrate ingredients for which it might be substituted. Comparative costs in Japan where trehalose has been sold since 1995 are as follows: Trehalose, $2.33-$2.50/kg; sucrose, $1.29/kg; high fructose corn syrup, $O.60-$O.67/kg; glucose syrup, $O.60/kg; dextrose, $O.93/kg, and maltose, $1.67/kg [120¥ = $1]. It is well known that the US food industry is one of the most cost sensitive sectors of the economy. Unless the technical benefits of trehalose are significantly greater than the increased cost, there is no advantage in its use.

2. Hayashibara trehalose is not expected to have desired technical advantages that would result in the wholesale replacement of added sugars in every food product. Many of the functional applications are similar or marginally advantageous to other more common, less costly sugars. Additionally, the functional properties of trehalose may not allow for the total substitution of a common sweetener with trehalose. For example:

   a) The concentration of trehalose needed to provide a desired technical effect may be cost prohibitive in certain cost sensitive food systems, such as commercial baked goods.

   b) Trehalose is 45% as sweet as sucrose. Therefore, trehalose may not deliver adequate levels of sweetness to food applications in formulations where it is totally replacing another sugar. Conversely, if used for its functional properties at levels that exceed the flavor threshold, the sweetness of trehalose may interfere with the flavor profile of a food system where sweetness is not desired.

   c) The physicochemical properties of trehalose are desirable in some food systems, but they do not lend themselves universally to all applications. For example, trehalose is a non-reducing

Section III Self-Limiting Levels of Use and Probable Consumption (Continued)

d) sugar, and resists carmelization. Reduction of browning is desired in many food systems,. However, in foods where browning is desired such as baked and fried foods, the addition of trehalose to a food formulation would not allow the final product to achieve a desired level of browning, unless it was included as part of a sweetening system with other reducing sugars.

e) Trehalose has the same caloric content as sucrose, and would not allow foods to be labeled as "sugar free", nor would it contribute to reduced calorie formulations.

f) Because it is a fully caloric sugar, trehalose will elicit a similar insulin response [Ruppin, *et a/.,* Vol 9 Tab 117]. Therefore, there may be no advantage to the use of trehalose in low-caloric or diabetic products.

Given these factors, Hayashibara feels it is reasonable to assume that certain self-limiting levels on the use of trehalose would occur in various food formulations. These economic and technical limitations are already being seen with the use of trehalose in Japan. US food formulators can be expected to go through a similar learning curve, as market development begins in this country.

B. Probable Consumption

1. Hayashibara trehalose as a food ingredient can be used in a wide variety of products ranging from traditional Japanese confectionery'to beverages, noodles, fruit purees, processed meat products and processed 'seafoods. Trehalose can also be substituted for a substantial portion of the sugars present in a given product. Therefore, it is likely that trehalose will be classified as a multiple-use direct additive, and as such could comprise a substantial portion of total product weight. Like other carbohydrates, trehalose is expected to be consumed as a macronutrient.

Consumption as a macronutrient may seem to represent an unusually high intake for Hayashibara trehalose given the fact that trehalose is consumed in relatively small quantities in most natural foods today. However, the actual intake may not be higher than historical consumption of trehalose by early man. Most assuredly early man consumed a large quantity of fungi, insects, and invertebrates as did his hominid ancestors [Eaton *et a/.*, 1988, Vol 5; Tab 47 and Diamond, 1992, Vol 5 Tab 44]. Indirect evidence for this can be found in the

Section III Self-Limiting Levels of Use and Probable Consumption (Continued)

relatively high trehalase activity found in the human intestinal tract [Friedman, 1978, Vol 5 Tab 54; Gould, 1995 a &b, Vol 6 Tabs 57 &58; Jones, *et al.,* 1992, . Vol 7 Tab 72]. Therefore, the human intestinal tract is already adapted to trehalose consumption. The digestibility of trehalose by humans will be discussed in more detail in a later section.

2. Hayashibara provided the Expert Panel an analysis of the maximum potential exposure levels to trehalose in accordance with FDA's guidance document entitled "Determination of Potential Exposure Levels for Food Ingredients". Use of the model described in the guidance document produced unreasonably high mean and 90th percentile intake levels for this' particular product. These intake levels were refined and mitigated in the trehalose GRAS Report and in discussions with the Expert Panel. The Expert Panel considered such facts as the amount of trehalose consumed *per capita* in the Japanese market today. Hayashibara trehalose is being used at high levels in rice formulations, which are traditionally consumed in Japan, in much higher amounts than are common in the US diet. The Panel concluded that there was sufficient safety data available on trehalose intake to support an exposure limited only by current Good Manufacturing Practice use levels in foods.

Within this Notification, Hayashibara wishes to provide a realistic assessment of the' probable consumption levels for Hayashibara trehalose, This realistic assessment is based on a more detailed analysis of the levels of use of trehalose in recognized food subcategories [as utilized by Market Research Corporation of America (MRCA)]. The use levels as a percentage of the total weight per recognized serving size [21CFR § 101,:12, Table 2: *Reference Amounts Customarily Consumed Per Eating Occasion: General Food Supply]* were determined from application development data across a wide variety of food categories, and bY,extrapolating use levels in the Japanese market to similar foods found in the US. Percentages of use in the various subcategories were summed, and then divided by the number of subcategories within each major food category (e.g., Baked Goods). This method produced an average percentage use level for the 43 food categories recognized by FDA at 21 CFR §170.3 (n). A table listing realistic percentages of use for Hayashibara trehalose in the United States is shown below.

Section III Self-Limiting Levels of Use and Probable Consumption (Continued)

Table 11
Realistic Use Levels of Hayashibara trehalose
In the Food Categories Listed by the US FDA At 21 CFR §170.3 (n)

	Food Categories	Realistic Percentage of Use in the US Market*
01	Baked Goods, Baking Mixes	3.47%
02	Beverages alcoholic	0
03	Beverages and beverage bases, non-alcoholic	2.92%
04	Breakfast Cereals	1.15%
05	Cheeses	0.66%
06	Chewing Gum	13.6%
07	Coffee and tea**	1.44%
08	Condiments and relishes	5.65%
09	Confections and frostingS	11.25%
10	Dairy product analogs	2.5%
11	Egg Products	3.125%
12	Fats and oils	1.0%
13	Fish Products	2.3%
14	Fresh eggs	0
15	Fresh fish	0
16	Fresh fruits and fruit iuices	4.17%
17	Fresh Meats	2.0%
18	Fresh poultry	0
19	Fresh vegetables	0
20	Frozen dairy desserts and mixes	10.0%
21	Fruit and water ices	2.0%
.22	Gelatins, puddings and fillings	10.62%
23	Grain products and pastas	2.33%
24	Gravies and Sauces	5.0%
25	Hard candy	10.0%
26	Herbs, seeds, spices, seasonings, blends	4.29%
27	Jams and iellies, home prepared	0
28	Jams and iellies commercial	10.0%
29	Meat products	1.81%
30	Milk, whole and skim	0
31	Milk products	4.13%
32	Nuts and Nut products	5.45%
33	Plant protein products	8.53%
34	Poultry products	1.73%
35	Processed fruits and fruit iuices	4.1%
36	Processed vegetables and vegetableiuices	3.86%
37	Snack foods	0.83%
38	Soft Candy	13.36%
39	Soups, home prepared	0
40	Soups and soup mixes	5.0%
41	Sugar, white granulated	0
42	Sugar substitutes	0
43	Sweet Sauces, toppings, and syrups	8.75%

*Based on Japanese commerclal use [Table 3, Sectton II, pages 20-23 of the Trehalose GRAS Report], US industry experience, and applications development.
**Coffee drinks containing 3% trehalose have now been commercialized in Japan.

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Section III Self-Limiting Levels of Use and Probable Consumption (Continued)

3. A second consumption table was prepared by multiplying the percentages of use of Hayashibara trehalose in various food subcategories by the mean consumption level reported for a single day, in USDA's food survey, *"Continuing Survey of Food Intakes by Individuals"(CSFII)* for 1996.

   The trehalose consumption from the various food categories is shown in Table 12 below, with a total mean intake of 34.43 grams per person per day given at the bottom of the table. Hayashibara believes the percentages of use are realistic for the US food industry.

Table 12
Estimated Intakes of Trehalose from Selected Food Categories
Based on CSFII, '96

Food Category	Use level (%)	Food Intake (g)	Trehalose Intake (g)
Baked goods, baking mixes			
Yeast Breads and Rolls	0	0	0
Quick breads, pancakes, etc.	3.47	20	0.694
Cakes, cookies, pastries, pies	3.47	38	1.32
Mixtures mainly grain	3.47	107	3.71
Breakfast cereals			
Ready-to-eat cereals	1.15	17	0.196
Grain products and pastas			
Rice	2.33	19	0.443
Pasta	2.33	21	0.489
Snack foods	0.83	31	0.257
Total Vegetables		132	
Fresh vegetables	0	0	0
Processed vegetables, juices	3.86	99	3.82
Total Fruits		162	
Citrus juices	4.1	59	2.42
Dried fruits	4.1	1	0.041
Non-citrus juices and nectars	4.1	26	1.066
Fruits and mixtures	4.1	18	0.738
Milk products			
Yogurt	4.13	8	0.33
Milk desserts	10.31	24	2.47
Cheese	0.66	16	0.106
Meat Products			
Sausages, processed meats	1.81	21	0.38
Mixtures mainly meat	1.81	99	1.79
Fish and shellfish (proc.)	1.81	5.5	0.1
Eggs			0.61
Egg Products"	0.03125	9	0.281

Section III Self-Limiting Levels of Use and Probable Consumption (Continued)

Table 12 (Continued)

Legumes			
Legumes	0.0549	28	1.53
Nuts and Nut Products			
Nuts and Nut Products	5.45	3	0.163
Fats and Oils			
Salad dressings	1	8	0.08
Total sugars and sweets			
Candy (Soft and Hard)	11.68	7	0.8176
Other Sugar Products[4]	11.12	15	1.67
Beverages Non-Alcoholic			
Coffee	0.0144	254	3.65
Teas	0.0144	128	1.84
Reg.Fruit drinks and ades	0.0417	82	3.42
Total Intake (grams)			34.4316

1 Processed vegetables were calculated by sUbtracting fresh vegetables from the total and multiplying the remainder by 0.75 to determine the consumption of vegetables likely to be processed with trehalose.

2. Hayashibara trehalose is unlikely to be used with fresh fish or shellfish. Therefore, the total amount consumed in this category was multiplied by 0.50 to represent the amount of fish or shellfish that might be consumed as 'a processed product. Applications for Hayashibara trehalose in processed fish and shellfish products have been commercialized in Japan.

3, Hayashibara trehalose may be consumed in dried egg products or in prepared egg products such as omelets. Therefore, the quantity consumed in this product was multiplied by 0.50 to represent use in such products.

4. This category contains such products as jams, jellies, sweet sauces, syrups, etc.

However, the estimated mean intake shown in Table 12 above still provides a conservative determination of potential mean consumption levels, since the following factors are assumed in the calculation:

a) Hayashibara trehalose is present in every product within a category.
b) All consumers are assumed to be "eaters only"
c) All eaters consume all the products in a given day.

In the agency's guidance document mentioned above, notifiers are allowed to assume that the 90th percentile of intake will be equivalent to approximately double the mean. If the potential mean consumption

Section III Self-Limiting Levels orUse and Probable Consumption (Continued)

level is 34.43 grams per day, then the 90th percentile would be equal to approximately 68.86 grams per day.

These values are still below the level of intake determined for sucrose by the NHANES III study [NHANES III, 1988-1994]. In that study the estimated mean intake for sucrose was 54.9 grams per day, and the 90th percentile was 110.7 grams per day. Therefore, even given the very conservative estimate for Hayashibara trehalose shown above, levels of trehalose use for all applications are expected to remain well below those for sucrose.

Additionally, several factors mitigate the 34.43 gram mean value:

a) As an example, Hayashibara is the only manufacturer of trehalose from. starch in the world. Since 1995 approximately 40,000 metric tons of Hayashibara trehalose were produced and sold into the Japanese market, predominately, but not exclusively for food. If the Japanese population (126 million) consumed this entire quantity in a one year period, the mean intake would only equal 0.317 kg per person per year, making the daily intake 0.870 grams per day (0.317 kg/365 days). This figure would translate into a 90th percentile equal to 1.740 grams per day person.

b) Another comparison can be made using the total sugar market in Japan for 1998. During that year, approximately 12,000 metric tons of Hayashibara trehalose was sold, while 4,343,000 metric tons of other similar sweeteners were sold. Trehalose consumption was 0.28% of these other sugars, and specifically 0.5% of sucrose. As discussed above, trehalose is substantially more expensive than other sugars. Even if the percentage of trehalose use in products increased about 180 fold over the current use level, the amount of trehalose would still be less than 50% ofthe total sugars consumed in Japan. In the U.S., the total consumption of all sugars in the diet, exclusive of lactose, was estimated in 1986 to be 80 grams/day, with the 90th percentile being 139 grams/day, according to the FDA [Glinsman, *et al.,* 1986 Tab 56, Vol 6].

From these data it was shown that of the 80 grams/day, approximately 53 grams/day represented all sugars (except lactose) that are added to the diet. Therefore, even if Hayashibara trehalose replaced all added sugars in the diet, the maximum mean would have been less than 53 grams/day (104 grams/day for 90th percentile), using 1986 data. If Hayashibara

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trehalose was used to substitute for 50% of all sugars added to the diet, the mean would constitute 26.5 grams/day, with the 90th percentile being 52 grams/day. However, the use of even this amount of trehalose seems highly unlikely for the reasons stated at the beginning of this section.

4. Given these factors, Hayashibara feels it is reasonable to assume that the daily exposure level for trehalose would be substantially less than the consumption of all sugars added to the diet, or of sucrose as reported by the Glinsman, 1986 and NHANES III studies [Glinsman, 1986 Tab 56, Vol. 6, and NHANES 1111988-1994].

   Hayashibara submits that there is a sufficient margin of safety to support the consumption levels presented above. All feeding studies conducted on mice, rats and rabbits have demonstrated a no-adverse-effect-level (NOAEL) of trehalose at levels up to 10% of the diet. Additionally, there have been several reports in which 50 grams of trehalose dissolved in water; have been consumed on an empty stomach by control subjects of Western European, ethnic background. In none of these instances was it reported that the subjects experienced adverse reactions. Other studies using mixed populations have demonstrated that trehalose is well tolerated on an empty stomach at levels in the tens of grams. These data are discussed in detail in Section IV. Finally, in excess of 80,000,000 pounds of Hayashibara trehalose has been sold into the Japanese market since late 1995. No reports of any consumer complaints have been made regarding this product.

Notification Section
V

Section IV. Detailed Summary of the Basis for the Determination that Hayashibara Trehalose is GRAS

A. Introduction

Hayashibara International Inc. has determined that the use of Hayashibara trehalose as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS as specified by 21 CFR §170.30. The GRAS determination was carried out under scientifIc procedures.

B. Detailed Summary

A detailed summary of the data and information which was considered as the basis for the GRAS determination is provided below in Section IV.

12. 1. Data Relied on to Establish Safety

Hayashibara compiled an eighteen-volume report, which supported the eligibility of Hayashibara trehalose as a GRAS ingredient in accordance with 21 CFR 170.30. The Hayashibara report included:

a) A description of the chemical identity and quantitative composition of Hayashibara trehalose;
b) Duplicate analyses of 5 lots of the product manufactured over approximately a six month period;
c) A description of the manufacturing process and quality control program;
d) A statement on the safety of the enzymes used to manufacture Hayashibara trehalose submitted by an expert in the field of enzyme safety and evaluation;
e) Final product specifications for Hayashibara trehalose, and
f) The physical properties of naturally occurring trehalose isolated from various sources, with a comparison to the Hayashibara trehalose prodUCt.

The report also presented data and information on the use and functionality of trehalose:

g) The historical" consumption of trehalose;
h) The content of Hayashibara trehalose in commonly eaten foods in Japan;
i) Estimates of the current consumption of Hayashibara trehalose in Japan; and
j) Estimates of the potential consumer exposure to Hayashibara trehalose in the United States.

k) A nutritional analysis of trehalose was presented;
l) Data describing the physicochemical (functional) properties relevant to its use as a food ingredient, and
m) Various developmental and commercial formulations containing Hayashibara trehalose were provided to demonstrate the technical effects of the ingredient in foods.

The report also included information concerning:

n) Results of several acute and chronic toxicological studies conducted in mice, rats, rabbits, and beagle dogs;
0) The metabolism of trehalose in the human body by the enzyme trehalase;
p) The published results of trehalose tolerance tests in humans.
q) A literature review of trehalase deficiency in individuals, showing that this condition appears to be rare in western populations.

2. Exposure

In estimating exposure to Hayashibara trehalose, the company relied on current use data from the Japanese market place, and on USDA's *Continuing Survey* of *Food Intake by Individuals,* which provided daily consumption figures by major food categories for the US population [Enns, *et a/.,* 1997, Vol 5 Tab 51]. An extremely conservative approach was taken in estimating exposure in the GRAS Report, in that maximum use levels for each food category were utilized. The maximum use levels were those, which were commercialized in processed foods in the Japanese market during the years 1995-1997. The levels were reported to Hayashibara by purchasers of the Hayashibara trehalose ingredient, or were from estimated consumer information. Maximum use levels for the Japanese market are shown for each major food category in Table 13 below.

The Expert Panel commissioned by Hayashibara based their estimates of total trehalose intake on data generated by the *Continuing Survey of Food Intake by Individuals.* The Panel suggested that the category of baked goods and other grain based products be divided into two separate categories. The suggested division was based on the fact that a high level of use of Hayashibara trehalose (up to 20%) occurs in certain commercial rice dishes in Japan. The Japanese population consumes much larger quantities and a greater variety of rice products than do North Americans. Using a maximum use level of 20%, across all foods in the baked goods and other grain-based food category

would skew the results in an unrealistic manner, when applied to a US population. However, the fact that a high level of Hayashibara trehalose has been consumed in a food category with a high average daily intake in Japan, provides evidence that trehalose is well tolerated in that population (below).

Several factors were evaluated by the Expert Panel including cGMP levels in Japan in order to reach the conclusion that exposure to trehalose would pose no threat to the consumer.

a) Hayashibaia trehalose is now formulated into hundreds of food products in the Japanese market.
b) The Japanese consumer has ,multiple governmental and commercial channels through which to report safety-related problems with food products'. Japanese consumers are known to use these mechanisms to report food-related safety issues [Japanese Consumer Reporting Procedures, Letter to Dr. Alan Richards, 1999 Vol 18 Tab 151].
c) Since the introduction of Hayashibara trehalose to the Japanese market in 1995, tens of millions of pounds have been sold to the food processing industry. Hayashibara has received no reports regarding a safety-related problem with Hayashibara trehalose.
d) Hayashibara trehalose may not offer technical advantages over other products that would offset economic considerations in many potential applications.
e) Adequate safety data exists from animal studies, published human tolerance studies, and Japanese. consumption patterns , to support human consumption of Hayashibara trehalose.

Based on a critical review of the data and information considered, the Expert Panel concluded that, Hayashibara trehalose could be used generally in foods at levels not to exceed those needed to produce an intended technical effect under current Good Manufacturing Practices.

Section IV Summary of Basis or GRAS Determination

Table 13
Current Good Manufacturing Use Levels
For
Hayashibara Trehalose in Foods·

Food Category1	Use Level2 (maximum)
Baked Goods	10%
Rice and Other Grain-Based Dishes	20%
Vegetables and Vegetable Dishes	5%
Fruits and Fruit Preparations	5%
Milk & 'Cheese	15%
Meat, Poultry and Fish	10%
Eggs and Egg Dishes	10%
Legumes and Legume Dishes	10%
Sugars and Sweets	60%
Coffee	3%
Fruit Drinks and Ades	5%

*Based on curren.t use levels of Hayashlbara trehalose in Japan.

1. Major food categories are based on those used in USDA's *Continuing* Survey of Food Intake by Individuals with rice and other grain-based dishes shown as a separate category [Enns, et *a/.,* 1997, *Family Economics and Nutrition Review 10:(4)].*
2. Maximum use levels are those which have been commercialized in processed foods in the Japanese Market during the years 1995-1997 as reported to the Hayashibara International by purchasers of the Hayashibara trehalose ingredient.

Section IV Summary of Basis of GRAS Determination

3. Technical Effects

Hayashibara trehalose is a multiple-use direct additive to food. It has a number of technical effects in food, which have been described in peer reviewed journals [Colaco *ef al.,* 1994 Tab 34 Vol 4, Roser, 1991b Tab 114 Vol 9, and Zappa, 1997 Tab 146 Vol 18]. In addition, Hayashibara International, Okayama, Japan and its customers have developed a number of other applications for trehalose.

In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (0) (1-32). Applications for trehalose are covered under several of the following terms as listed under 21 CFR §170.3 (0).

(4)"Colors and coloring adjuncts": Substances used to impart, preserve, or enhance the color or shading of a food, inclUding color stabilizers, color fixatives, color-retention agents, etc.

(11)"Flavor enhancers": Substances added to supplement, enhance or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.

(16)"Humectants": Hygroscopic substances incorporated in food to promote retention of moisture, including moisture-retention agents and antidusting agents.

(21)"Nutritive sweeteners": Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

(28)"Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.

(31)"Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.

(32)"Texturizers": Substances, which affect the appearance or feel of the food.

Examples of the ability to provide these technical effects can be found in the Japanese market place, and applications development is well underway in the US.

4. Contaminants and Manufacturing

Hayashibara has developed and patented a novel process for producing trehalose enzymatically from starch Section II that Hayashibara was the first company in the world to commercialize the enzymatic conversion of starch to dextrose in 1959. Since that time,

Section IV Summary of Basis of GRAS Determination

Hayashibara International has remained a leader in the enzymatic conversion of starch to many useful saccharide-based products. These type of products have likely been sold in every country in the world as food ingredients, and the process upon which Hayashibara trehalose is based, is used by all major sweetener-producing companies around the globe. The trehalose product obtained from the Hayashibara process is currently approved by the appropriate regulatory agencies, and is being sold in Japan, Taiwan and Korea as a food ingredient or additive.

All raw materials used in the process are food grade in Japan, and Hayashibara provided specifications for those raw materials in its GRAS Report. It is understood, that if production of Hayashibara trehalose occurs in the United States, all raw materials will meet US food grade standards.

The Hayashibara technology includes the use of seven enzymes, two of which are novel trehalose-producing enzymes discovered by Hayashibara scientists. All the substances used in the manufacturing of trehalose are used in accordance with current Good Manufacturing Practices (21 CFR 110.80(a).

As described in Section II, one of the final steps in the production of trehalose, involves crystallizing the sugar out of a trehalose-rich solution. Because crystallization is inherently a purification step, it is unlikely, that contaminants are going to be associated with the final food product. The specifications presented in Section II, along with the analyses of five lots in duplicate support this conclusion. The product produced by this mechanism is in the dihydrate form.

Data was presented to the Expert Panel, which demonstrated that all the enzymes used to produce Hayashibara trehalose were denatured during the course of processing. Of the seven enzymes used, four are commercially available and have EC numbers assigned (See raw materials list above). The Hayashibara International commissioned Dr. Michael Pariza, Director of the Food Research Institute at the University of Wisconsin to prepare an expert opinion regarding the safety of the novel enzymes used in the process. Dr. Pariza found no safety concerns associated with any of the enzymes, and his report is attached to this notification. Mr. Cleve Denny, a member of the Expert Panel and noted food microbiologist, also provided a review of the safety of the enzymes.

In conclusion, Hayashibara trehalose, has a high level of purity, and the company has well documented process controls in place.

Section IV Summary of Basis of GRAS Determination

The results of analyses of 5 lots in duplicate manufactured over a period of time demonstrated that the production process is capable of meeting the defined specifications on a consistent basis.

5. Safety Studies

not published

a) Several preclinical studies have been performed using trehalose. A few of the original studies were done by Quadrant Holdings using a trehalose preparation that was approved in the UK as a novel food for use as a cryoprotectant [European Biotechnical Newsletter, 1991 Vol 5 Tab 52]. The bulk of the preclinical studies were commissioned by Hayashibara to assure the safety of trehalose and their unique production technology. Testing included standardized *in vitro* assays, as well as oral dosing of Hayashibara trehalose to rodent and non-rodent species. Analyses of these studies consistently showed that trehalose demonstrated no oral toxicity at the levels tested. Table 14 below summarizes those results.
b) The three reproductive studies, in which the trehalose product was fed at levels up to 10% of the diet, were conducted with Expert Panel direction and essentially in accordance with the 1982 edition of the FDA handbook on *Toxicological Principles,* and current Organization for Economic Cooperation and Development (DECO) guidelines. These three studies included a two-generation reproductive study in rats, an embryotoxicity/teratology study in rabbits, and an embryotoxicity/teratology study in rats.
c) No adverse effects or untoward observations were seen, even when Hayashibara trehalose was fed at levels up to 10% of the diet. These data indicate that Hayashibara trehalose is well tolerated by a variety of mammalian species and caused no toxicological effects, in any studies, even at the highest doses tested.

Section IV Summary of Basis of GRAS Determination

Table 14
Hayashibara Trehalose Safety Studies

Test Material	Test/Species	Study Type	Route	Dose	Results
Hayashibara Trehalose	S. *typhimurium* and *E. coli*	Bacterial Mutation Assay	*In Vitro*	5,000 μg/plate	No evidence of mutagenic toxicity
Hayashibara Trehalose	CHOCells	Chromosome Aberration Assay	*In Vitro*	5,000 μg/ml	No Mutagenic Activity
Hayashibara Trehalose	Micronucleus Assay	Mice	IP	*5,000 mg/kg*	No Toxicity
Quadrant Trehalose	Albino Mice	Acute Toxicity (1 dose)	Oral by gavage	5g/kg	No Toxicity
Quadrant Trehalose	Albino Rats	Acute Toxicity (1 dose)	Oral by gavage	*5g/kg*	No Toxicity
Hayashibara Trehalose	Beagle Dogs	Acute Toxicity	Oral by capsule	*5g/kg*	No Toxicity
Quadrant Trehalose	Albino Rats	Acute Toxicity (1 dose)	Oral by gavage	*16g/kg*	No Toxicity
Quadrant Trehalose	Beagle Dogs	14 Day	Oral by capsule	*5g/kg/day*	No Toxicity
Quadrant Trehalose	Albino Mice	14 Day	Oral by gavage	*5g/kg/day*	No Toxicity
Hayashibara Trehalose	Albino Mice	13 Week	Oral in diet	*8.3g/kg/day* (aver. of sexes) NOAEL*	No Toxicity
Hayashibara Trehalose	Wistar Rats	Embryotoxicityl Teratogenicity	Oral in diet	The NOAEL was the mean of the highest level tested 6.94 ± 1.61 g/kg/d	No Toxicity
Hayashibara Trehalose	New Zealand White Rabbits	Embryotoxicityl Teratogenicity	Oral in diet	The NOAEL was the mean of the highest level tested 1.99 ± 0.43 g/kg/d	No Toxicity
Hayashibara Trehalose	Wistar Rats	Two-generation Reproductive	Oral in diet	The NOAEL was 7.09 ± 0.45 *g/kg/d* for males, and 6.16 ± 0.48 *g/kg/d* for females	No Toxicity

*The laboratory which performed this study used the term NOTEL (No-Toxlc-Effect-Level)

Section IV Summary of Basis of GRAS Determination

6. Human Safety

The Hayashibara GRAS Report compiled and reviewed information from published studies, which revealed that the ability to digest trehalose evolved much earlier than did hominid characteristics. Trehalose was likely a relatively high portion of prehistoric man's diet, and the recorded history of human consumption of trehalose may extend to early Biblical times. The presence in the human intestinal system and kidneys of relatively large quantities of an enzyme (trehalase) that specifically cleaves trehalose into two glucose units strongly suggests that humans have the capacity to safely consume trehalose at relatively high concentrations. This physiological mechanism is consistent with other commonly digestible disaccharides, such as sucrose, maltose or lactose, which are also hydrolyzed to monosaccharides prior to absorption.

It is believed that trehalose intolerance occurs substantially less frequently than lactose intolerance. Only a few reports are found in the literature. However, the reader should note that several specific studies have been performed to identify its prevalence. Information on the frequency of intolerance to isolated trehalose has come from three sources: human tolerance tests, enzyme (trehalase) assays of biopsy specimens, and recent consumption data from Japan. Hayashibara GRAS Report contained a review of the literature on human trehalase activity and on human trehalose tolerance tests. Noteworthy citations are summarized below and in Table 15. Since there were no toxicologically significant adverse effects revealed in the animal studies discussed above, and since several studies demonstrated that 50 gram bolus doses of trehalose could be tolerated by the vast majority of Caucasian populations, Hayashibara reviewed the few studies available on trehalose intolerance in more detail, in order to place those studies in perspective with other reports, where no effect of trehalose ingestion was observed. Reviews of those studies appear below. It is important to note that low values for enzyme activity are highly correlated, but not an absolute predictor of disaccharide intolerance [Dahlqvist, 1974 Tab 41 Vol 5].

a. Trehalase Prevalence

It has been suggested that trehalase malabsorption may occur when trehalase enzymatic activity is 6.0 IU/g protein or less, while intermediate levels would be 6.0 to 8.0 IU/g protein. Gudmund-H0yer, *et al.* referred to a study of trehalase activity of intestinal biopsies from 248 Danish patients in which the

lowest level of activity in the group was 8.3 IU/g and the median activity was 31 IU/g. The authors reported the corresponding figures for lactase activity as 6.1 IU/g and 32 IU/g respectively [Gudmund-H0yer, *et al.,* 1988 Tab 60 Vol 6].

In a study of 100 consecutive normal biopsy samples from adults in Switzerland (72 male, 28female), two subjects with low trehalase activity (2.7 and 1.5 IU/g) were identified [Bergoz, *et al.,* 1973 Tab 14 Vol 4]. The investigators suggested that a trehalase value < 5 IU/g might result in trehalose intolerance. This conclusion is in basic agreement with that of Gudmund-H0yer, *et al.,* who also stated stated that in more than 500 biopsies of Danish subjects, no trehalase deficiencies had been observed [Gudmund-H0yer, *et al.,* 1988 Tab 60 Vol 6].

Welsh described intestinal trehalase activity in 123 Caucasian subjects from the southwestern United States, ranging in ages from 1 month to 93 years [Welsh, *et al.,* 1978 Tab 138 Vol 13]. The lowest recorded values were 7 and 8 IU/g in two groups of infants 0 to 1, and 1 to 2 years of age (n=70), respectively. No statistically significant differences in trehalase activity were found between any age group or by gender. Importantly, trehalase activity did not appear to be lower in infants, or to wane with age. Finally, no correlation was seen between lactase and trehalase activity.

Twenty patients in Czechoslovakia with no bowel symptoms or related disease were examined for trehalase activity [Madzarovova-Nohejlova,et *al.,* '1973 Tab 85 Vol 8]. None of these subjects were considered to be trehalase deficient.

Murray *et al.,* reported on intestinal trehalase activity from 369 biopsies obtained from subjects in the U.K. (Murray *et al., 2000 British Journal of Nutrition* 83: 241-245). Only one subject (0.3%) was shown to have low trehalase activity (4.5 U/g protein). The single subject was considered just below the normal range.

Thirty-five (35) Finnish subjects were tested for trehalase to determine enzymatic activity [Asp, *et al.,* 1971 Tab 5 Vol 3]. Nineteen of the subjects were lactase deficient. The mean enzymatic activity of trehalase was 28.3 *U/g* (± 8.6) with a range of 13 to 46 U/g. There was no correlation between lactase and trehalase activity.

Mališ and coworkers studied jejunal biopsies of patients with celiac disease and mucoviscidosis for trehalase activity (Malis F., *et al.,* 1972, *Digestion,* 5: 40-48). Included in the study was a control group consisting of 15 adults and 17 children. Mean (± SD) activities for each group were 33.3 ± 3.5 and 28.5 ± 4.9 *U/g* protein, respectiveiy. No trehalase deficiencies were noted in the control subjects.

Jonsson *et al.,* took samples from two areas of the duodenum and examined disaccharidase activities. The purpose was to examine the differences in activity between samples from the same and different areas of the duodenum. In the ten biopsies from the middle of the duodenum, the mean enzymatic activity was 11.5 ± 5.5 protein. While this was considered low, all other levels of disaccharidases tested by the authors were lower than those reported by others. Murray *et al.,* in his paper considered these values normal (Jonsson *et al.,* 1986 *Scandinavian Journal of Gastroenterology,* 21: 51-54).

In the same report Murray *et al.* presented summarized data from three other studies·from Sweden, Denmark, and Switzerland, in which a total of 231 subjects were assayed for trehalase. The authors concluded that in none of the subjects was there a trehala.se deficiency (Murray *et al.,* 2000 *British Journal of Nutrition* 83: 241-245).

A review of the literature, as summarized in Table 15 below, shows that trehalase activity in humans has been assayed by a number of authors over a period of several decades. Out of more than 1400 biopsies reported in the literature, only three samples were shown to be deficient in trehalase and two others had levels thatwould be considered intermediate (6-8 IU/g protein or less). From the published results, it appears that when trehalase activity assays are performed on hundreds of control subjects from a western Caucasian population, only a few individuals could be identified with low trehalase activity. Importantly, this percentage «0.1 %) appears substantially less than the percentage of persons with lactase and possibly other disaccharidase deficiencies [Dahlqvist, 1974 Tab 41 Vol 5].

Based on the accumulated data, one can conclude that the human body is well equipped with the enzymatic system necessary for trehalose digestion, and that the few cases of low

Table 15
Human Trehalase Activity

Number of Biopsies	Location	Results	Citation
500	Denmark	No trehalase deficiencies observed	[Gudmund-H0yer, *et al.,* 1988 Tab 60 Vol 6]
123	Southwestern U.S.	No trehalase deficiencies. Two neonates had low levels; no significant differences between age groups; Enzyme activity did not diminish with age. Trehalase normal in lactase deficient subiects.	[Welsh, *etal.,* 1978 Tab 138 Vol 13]
13	Denmark	No trehalase deficiencies observed	(Murray *et al., 2000 British Journal of Nutrition* 83: 241-245).
100	Switzerland	Two with low trehalase activity	[Bergoz, *et al.,* 1973 Tab 14 Vol 4]
20	Czechoslovakia	No trehalase deficiencies observed	[Madzarovova-Nohejlova, *et al.,* 1973 Tab 85 Vol 8]
35	Finland	No trehalase deficiency. Several subjects lactase deficient	[Asp, 1974 *et al.,* Tab 5 Vol 3]
32	Czechoslovakia	No deficiencies in 15 adults and 17 children	(Malis F., *etal., 1972, Digestion,* 5: 40-48)
369	U.K.	One subject with low trehalase . activity (0.3%)	Murray *et al.,* 2000 *British Journal of Nutrition 83:* 241-245).
10	Sweden	No trehalase deficiencies	(Jonsson *et al., 1986 Scandinavian Journal of Gastroenterology,* 21: 51-54).
231	Sweden Denmark and Switzerland	No trehalase deficiencies (Taken from review by Murray *et al.,* 2000*)	Murray *et al.,* 2000 *British Journal of Nutrition 83:* 241-245).

*Murray, et *al.,* 2000. Intestinal Trehalase ActiVity in a U.K. PopulatIon: Establishing a Normal Range, and the Effect of Disease, *British Journal of Nutrition,* 83: 241-245.

b. Trehalose Consumption

In addition to the data available on the presence and concentration of trehalase in a general population, there are also reports on the consumption of large amounts of trehalose by various populations.

In three separate studies, control subjects were given 50 grams of trehalose in 400 ml of water as a bolus. This was administered to fasting subjects, which would provide a worst case scenario for an individual's ability to assimilate a large amount of a disaccharide. In each of the studies, consisting of a total of 126 subjects, all subjects were reported to tolerate the dose given [Bergoz, 1971, Tab 13 Vol 4, Bergoz, *et al., 1973* Tab 14 Vol 4, and Bolte, *et al.,* 1973, Tab 149 Vol 18].

In two recent studies from Japan, it could be inferred that Asian populations, at least Japanese, may have a slightly lower threshold for trehalose ingestion. In the first study, written in Japanese, thirty subjects were used (15 female, 15 male) including 10 Mongoloid (Japanese), 10 Caucasians, 8 African-American, and 2 designated as other [Ushijima, *et al.,* 1995 Tab 135 Vol 12]. Subjects were fasted overnight and the subjects were given 10, 20, 30 and 40 gram doses of trehalose. Blood glucose concentrations and hydrogen (or methane) gas expiration was measured before and every 30 minutes after trehalose ingestion for 3 hours. An increase of 20 ppm of hydrogen (or 1 ppm methane) was considered to be a sign of malabsorption. Malabsorption rates of 0, 40, 43 and 75% were observed when subjects ingested 10, 20, 30 and 40 grams, respectively. Gastrointestinal (GI) symptoms were also observed, with occurrences of 0,40,43, and 50%, respectively. No breakdown was reported as to the response according to racial groupings, the severity of the malabsorption, or the type and severity of the GI symptoms.

In a second experiment using the same group of subjects, 0.6 grams of trehalose per kg body weight was administered. The differences in malabsorption rates, as assessed by hydrogen gas expiration, were not significant between the racial groups (Mongoloid =50%, Caucasian =67%, African-American =63%). Interestingly, Mongoloid subjects had dramatically higher GI symptoms (90%) than either the Caucasian (11%) or African-American (0%) subjects. Additionally, blood glucose concentrations (30 minutes) were significantly higher in

Caucasians (37.6 ± 11.1 *mg/dl)* and African-Americans (24.3 ± 10.3) than Mongoloid subjects (11.0 ± 8.6). Although the data presented are sketchy at best, it suggests that when equal amounts of trehalose are given on a body weight basis, Caucasians and African-Americans appear to be able to absorb glucose at a significantly higher rate than Mongoloid subjects. It also appears that the use of hydrogen gas expiration is not consistent with increases in blood glucose, or symptom assessment. Because of the lack of specific details and consistency of results, conclusions drawn from this study will need to be examined against other work.

In the second study trehalose and lactulose tolerance was measured on young, healthy Japanese women [Oku, *et al.,* 1998 Tab 105 Vol 9]. Doses of 30,40,50, and 60 grams of trehalose in 200 ml water were given to each subject 2 to 3 hours after eating. Subjects ingested the next greater amount of saccharide until they experienced diarrhea. Subjects recorded the time of onset and type of abdominal symptoms (if any), and stool frequency and consistency. No subjects had diarrhea when given 30 grams of trehalose. At 60 grams of trehalose, 10 of the 20 subjects had reported diarrhea. Abdominal symptoms were reported in subjects at the 30 gram dose. These included: nausea (10%), discomfort (15%), flatus (40%), distension (20%), borborygmus (45%), and low abdominal pain (5%). The symptoms became progressively more prevalent as the dose increased. The severity of each symptom was not reported.

The authors calculated the transitory laxative threshold of trehalose as 0.65 *g/kg* body weight using regression analysis *(0.28 g/kg* for lactulose). This calculates to a dose of 33 grams for a person weighing 50 kg, which was the average of the study population. The authors made the point that this study used a single large dose of trehalose, given as a bolus. It has been shown that splitting doses between several eating occasions can increase the ability of the system to digest similar substances. It was also concluded that, as with tolerance to other disaccharides, it is likely that colonic adaptation to regular ingestion of trehalose will take place, which would increase the amount that a person could ingest (Hertzler and Savaiano, *1996. American Journal of Clinical Nutrition,* 64: 232-236). It is known that when disaccharides (trehalose) are used in a food product, they are digested over a longer time period, thus allowing for more complete digestion [Elias, *et al.,* 1968 Tab 50 Vol 5]. Finally, the dose of trehalose administered was given in

Section IV Summary of Basis of GRAS Determination

200 ml of water, rather than the more common 400 ml. This would increase the osmotic strength of the solution by two-fold. It is possible that the seemingly lower level of tolerance was related not only to the quantity of trehalose consumed, but also to the osmolarity of the solutions.

Taken at face value, it appears from the data presented that Mongoloid (Japanese) populations may have a slightly reduced capacity to absorb trehalose; however, the amounts that are digestible appear to be relatively large. In a total of 30 Mongoloid subjects a single dose of 30 grams was well tolerated. In the 126 subjects from a predominately Caucasian population given a single dose of 50 grams of trehalose, no subjects reported abdominal problems. The first Japanese study showed that no abdominal symptoms were reported by the 10 African-Americans and only 1 of 9 Caucasians given 0.6 g/kg body weight of trehalose; the equivalent of 42 grams of trehalose for a,70 kg man reported symptoms. Ingesting trehalose at multiple times during a day, and including it in food products would likely raise the no effect dose level of trehalose considerably above the amounts suggested in these studies.

The relationship between intolerance to mushrooms, and trehalase activity and trehalose tolerance was recently examined (Arola *etal.,* 1999 *Sacandinavian Journal of Gastroenterology* 34: 898-903). Sixty-two (62) subjects were included. Thirty (30) subjects were selected because of their self-professed inability to eat mushrooms without symptoms of gastric distress. Thirty-two (32) control subjects reported no intolerance to mushrooms. All subjects were given 25 grams of trehalose dissolved in 400 ml of water as a bolus after fasting overnight. Blood glucose and breath hydrogen (or methane) concentrations were measured over time. Gastroscopy was performed to assess the amount of gastritis, and duodenal biopsies were obtained for trehalase assays. Subjects were scored for symptoms experienced after trehalose ingestion.

Of the 30 mushroom-intolerant subjects, only 19 had gastrointestinal symptoms after consuming the trehalose, and were termed "trehalose-intolerant". Eleven (37%) of the mushroom-intolerant group did not report any intolerance to trehalose. No subjects from the control (mushroom-tolerant) group were trehalose-intolerant. .No statistical differences between the two groups were noted in the incidence of gastritis, blood glucose concentration, or breath hydrogen (methane)

concentration. The mushroom-intolerant group had significantly lower (P<0.03) mean trehalase activity (21.4 ± 8.1) than the control group (29.1 ± 14.1). Subjects were then organized and analyzed as either trehalose-tolerant (n=43) or intolerant (n=19). The trehalose-intolerant group was further divided into those having mild (n=13) or severe (n=6) gastrointestinal symptoms.

Interestingly; statistical analysis revealed no significant differences in trehalase activity or blood glucose between the means of the trehalose-intolerant and tolerant groups (Arola *et al.,* 1999 *Sacandinavian Journal of* Gastroenterology 34: 898-903). There was a significant positive correlation (P<O.OS) between blood glucose-and intestinal trehalase activity. However, the mean trehalase activity of the trehalose intolerant group was less than that of the tolerant group, the range of trehalase activity for the trehalose-intolerant group was 10.6 to *34.2 U/g* protein. None of the "intolerant" subjects had deficient enzymatic activity. If 8 *U/g* protein is the level at which no symptoms are observed then none of these subjects should have exhibited symptoms.

Interestingly, two subjects in the mushroom-tolerant and trehalose-tolerant group were shown to have deficient trehalase activity. Both results are counterintuitive, and seemingly inconsistent with other reports [Bergoz, *et al.,* 1971 Tab 13 Vol 4, Bergoz, *et al.,* 1982 Tab 15 Vol 4, Gudmand-H0yer *et al.,* 1988 Tab 60 Vol 6, and Madzarovova-Nohejlova, 1973 Tab 85 Vol 8].

Jonsson *et al.,* showed that sampling of biopsy tissue can cause highly variable results in trehalase enzyme activity values (Jonsson *et al.,* 1986 *Scandinavian Journal of Gastroenterology,* 21: 51-54). It may be that the two subjects with low enzymatic values represent a sampling error, or conversely demonstrate the variability of enzymatic activity with malabsorption Dahlqvist, 1974 Tab 41 Vol 5].

One of the most important considerations, when evaluating the safety of trehalose ingestion, is the nearly 5 years of experience in selling Hayashibara trehalose into the Japanese market. In excess of 80 million pounds have been sold with no adverse effects reported.

Table 16 below lists studies and numbers of subjects that were subjected to trehalose tolerance tests.

Table 16
Tolerance of Trehalose

Number of Subjects	Location	Results
16	Switzerland	Tolerated 50 gram bolus
50	Switzerland	Tolerated 50 gram bolus
60	Germany	Tolerated 50 gram bolus
30	Japan/USA	0.6 g/kg given. Nine of 10 Japanese, 1 of 9 Caucasians, and a of 8 African-Americans reported GI symptoms. Japanese subjects absorbed less glucose
20	Japan	Author concluded 33 grams (0.6 *g/kg)* of trehalose as a bolus in Japanese women was tolerable. Some GI symptoms noted
62	Finland	Mushroom tolerant and intolerant subjects were given 25 grams of trehalose in 400 ml following an overnight fast. Thirteen of a trehalose intolerant group had mild symptoms, and 6 subjects reported severe symptoms.
	Japan	In excess of 80 million pounds of Hayashibara trehalose has been sold into the market in hundreds of food products. No reports of intolerance

c. Persons at Risk

An important safety consideration for any food ingredient is the populations which may be at risk following ingesting of the substance. It appears that the consumption of all disaccharides, including trehalose, may be of concern for three groups of individuals:

1) Those who lack the enzyme necessary for the digestion of the disaccharide, or have very low levels of enzyme activity, and are therefore intolerant,
2) Persons in certain ethnic populations, who may have a hereditary pattern of specific enzyme deficiency, and
3) Patients with intestinal malabsorption disorders, where there is a consistent correlation between the

severity of the morphological findings and reduced enzymatic activity [Berg *et al.,* 1973; Bergoz, *et al., 1973* Tab 12 Vol 4; Bolte *et al.,* 1973 Tab 149 Vol 18; Mališ F., *et al.,* 1972, *Digestion,* 5: 40-48; Murray *et al., 2000 British Journal* of *Nutrition* 83: 241-245; and Rodeck and Dominick, 1983 Tab 112 Vol 9].

The Hayashibara GRAS Report contained references to studies reporting malabsorption and/or intolerance to trehalose. Clinical symptoms appear to be identical to those seen in other disaccharide malabsorption syndromes, and are therefore self-limiting.

Within Caucasian populations, only a few individuals have been identified with low trehalase activity. Importantly, the number appears substantially less than lactase deficiency and possibly other disaccharidase deficiencies [Dahlqvist, 1974 Tab 41 Vol 5].

d. Reports of Trehalose Intolerance in Caucasians

The first suggested case of trehalase deficiency was reported by Bergoz in 1971 in a 71 year old Swiss woman [Bergoz, 1971, Tab 13 Vol 4]. She had noticed for at least 20 years that consumption of mushrooms provoked diarrhea. The author gave the patient 50 grams of trehalose in 400 ml of water. The patient experienced symptoms of bloating, abdominal cramping, and presented liquid stools starting 70 minutes after trehalose ingestion. Because of other health issues, a biopsy to test for trehalase activity was not possible.

MadzarovQva-Nohejlova *et al.,* reported the case of a 24-year old white man in Czechoslovakia who was admitted to University Hospital with vomiting and diarrhea resulting from the ingestion of mushrooms. It was later discovered that other members of his family also experienced mushroom intolerance. A trehalose tolerance test was performed in which 50 grams of trehalose was administered. Symptoms similar to those experienced after eating mushrooms were noted in the patient and his father. A complete absence of trehalase activity was found in both individuals. The conclusion was that an autosomal type of heredity for trehalase deficiency seemed likely (Madzarovova-Nohejlova, *et al.,* 1973 Tab 85 Vol 8].

e. Reports of Trehalose Intolerance in Ethnic Populations

Gudmand-H0yer *et al.* studied intestinal biopsies from 97 adult (50 female, 47 male) residents of Greenland with peptic ulcers, or cancers [Gudmand-H0yer *et al.,* 1988 Tab 60 Vol 6]. The trehalase enzyme activity in this population ranged from 0 to 94 *U/g* protein (median of 18 *U/g* protein). A Danish control population of 248 patients was shown to have a median trehalase activity of 31 *U/g* protein (lowest activity 8.3 *U/g* protein). Examination of individual Greenland subjects revealed that 8 subjects had trehalase values of less than 6 *U/g* protein, and another 6 had values less than 8 *U/g* protein. Three subjects with low trehalase activity « 6 *U/g)* were given 50 grams of trehalose dissolved in 250 ml water. No glucose was assimilated into the blood. The authors stated that a high incidence of trehalase deficiency in these populations was not surprising. Lactase deficiency is found in approximately 60% of Greenlanders. Additionally, sucrase deficiency, which is almost unknown among other ethnic groups, is not uncommon in Greenlanders IGudmand-H0yer *et al.,* 1988 Tab 60 Vol 6]. The high prevalence of disaccharidase deficiencies are likely associated with the native Greenland population (Inuits). This is demonstrated by lactose deficiency, where the prevalence decreases with the proportion of European (lower lactose deficiency) parentage (Gudmand-H0yer and Skovbjerg, 1996 *Scandinavian Journal of Gastroenterology* 31, Supplement 216: 111-121).

f. Persons With Metabolic or Digestive Disorders

Patients with juvenile diabetes have been tested for trehalase activity. Significant depression of trehalase activity was not seen, although the mean enzymatic activity of these patients was lower than controls [Ruppin, *et al.,* 1974 Tab 117 Vol 9]. It was suggested that the level of trehalase activity may be insulin-dependent. Cerda and coworkers reported that non-insulin dependent diabetic patients, with chronic pancreatic insufficiency, exhibit a 2-fold increase in trehalase and other disaccharidases [Cerda, *et al.,* 1972 Tab 31 Vol 4]. Patients with chronic renal failure appear to have reduced levels of trehalase, but the reduction appears to be less than for other disaccharides [Dennenberg, *et al.,* 1974 Tab 43 Vol 5]. Caspary et *al.* found that patients with chronic pancreatitis and exocrine pancreatic insufficiency had normal lactase and trehalase levels, whereas maltase and sucrase were elevated [Caspary et

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al., 1975 Tab 30 Vol 4]. The more common condition of lactase deficiency (lactose intolerance) is not correlated to a deficiency of trehalase in the general population [Asp, *et al.,* 1971 Tab 5 Vol 3].

Several authors have reported that all disaccharidase activities are significantly reduced in those individuals with untreated celiac disease. In that condition patients have been diagnosed with partial or total villus atrophy, and hence the site of secretion for the disaccharide enzymes has been compromised [Berg *et al.,* 1973; Bergoz, *et al.,* 1973 Tab 12 Vol 4; Mališ F., *et al., 1972, Digestion,* 5: 40-48; Murray *et al.,* 2000 *British Journal of Nutrition* 83: 241-245].

While unlikely in the general population, the publications described above have indicated that trehalose intolerance is a rate limiting gastrointestinal condition which presents no chronic health concerns and can be controlled by simple restriction of the product from the diet of those few individuals who may be intolerant or become intolerant because of certain gastrointestinal diseases. These are the identical restrictions and interventions that would be used for any such medical condition.

C. Summary

An expert panel qualified by scientific training and experience to evaluate the safety of food and food ingredients was assembled to conduct an independent critical evaluation of the safety of Hayashibara trehalose as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein. The panel members included:

Dr. Roy L. Whistler, Chairman	Carbohydrate Chemist
Dr. Gary Flamm	Toxicologist
Dr. Joseph Borzelleca	Pharmacologist and Toxicologist
Dr. George Burdock	Toxicologist
Mr. Cleve Denny	Microbiologist

The qualifications of the members of the Scientific Expert Review Panel are provided in their curricula vitae, which are on file at the offices of Hayashibara International Inc., 2201 Civic Circle, Suite 719, Amarillo, Texas USA 79109. The Panel's qualifications in their respective scientific fields met the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201 (s» and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

The Expert Panel was charged by Hayashibara to critically evaluate the information and data regarding trehalose in general and Hayashibara trehalose specifically. The Expert Panel rendered an opinion, based on scientific principles, on the generally recognized as safe (GRAS) status of Hayashibara trehalose. The opinion of the Panel is based on the general use of Hayashibara trehalose in food when manufactured in accordance with U.S. current Good Manufacturing Practices (21 CFR Part 110) and meeting the specifications provided in the report.

Members of the Expert Panel independently critically evaluated the results of a literature review on trehalose, information provided by Hayashibara, and other pertinent materials. The Expert Panel conferred by telephone and then met in Atlanta, Georgia on November 17,1999 with representatives of Hayashibara, who reviewed the pertinent safety and functionality information associated with Hayashibara trehalose. This information was compiled into an eighteen-volume report, which supported the eligibility of Hayashibara trehalose as a GRAS ingredient in accordance with 21 CFR 170.30. The information provided for the Expert Panel's review was presented in the form stipulated in 21 CFR 170.35. The Expert Panel's written opinion is attached to this Notification.

Section IV Summary of Basis of GRAS Determination

Based on the Expert Panel's independent and collective critical evaluation of all the information summarized above, it was determined that Hayashibara trehalose can be considered generally recognized as safe (GRAS) when produced and used in accordance with cGMP for the various major food categories as listed by the United States Department of Agriculture in the *Continuing Survey* of *Food Intake by Individuals* (CSFII), and meeting the specifications described in Section II.

In reaching this conclusion, the Panel relied on the fact that:

1. The various acute and chronic safety studies conducted by Hayashibara in four (4) species of animals showed no detectable toxicity at the doses tested. These animal safety studies have demonstrated that Hayashibara trehalose is non-toxic to the animals consuming the substance or their unborn offspring during gestation
2. The published information shows that trehalase deficiency is rare in humans. Several published reports on trehalose intake by humans have revealed that, while a trehalase deficiency exists, trehalose is well tolerated in most populations. Two Japanese studies suggested that individuals of Mongoloid 'ethnic background may have a lower tolerance for trehalose than either Caucasian or African-American populations.
3. These reports are placed in perspective by the fact that since the introduction of Hayashibara trehalose in 1995, tens of millions of pounds of Hayashibara trehalose have been consumed in Japan without any food safety-related reported incidents.
4. Hayashibara trehalose is used in hundreds of Japanese products;
5. Japanese food manufacturers have utilized trehalose at relatively high concentrations (up to 20%) in products, such as rice dishes, which are known to be consumed more frequently in Japan than in the United States;
6. The Japanese consumer may be more sensitive to trehalose intolerance than Caucasian or African-American populations; and
7. No complaints have been received by the Hayashibara Company Ltd. or its distributors regarding Hayashibara trehalose, even though the Japanese consumer is known to frequently avail themselves of the procedures for reporting food-related safety issues. A description of the various reporting procedures available to the Japanese consumer is contained in the Hayashibara GRAS Report [Tab 151 Vol 18].
8. Hayashibara will publish the results of its safety studies in a peer-reviewed journal.

The Panel noted that:

9. Hayashibara trehalose is produced enzymatically from corn starch grown and produced in the United States, and other raw materials considered food grade in Japan using current Good Manufacturing Practices (cGMP);
10. The enzymes used to convert the starch into trehalose originate from non-pathogenic/non-toxigenic/non-GMO organisms; and
that the product has a high level of purity.
11. Hayashibara has provided final product specifications for its trehalose ingredient.
12. Based on the experience in the Japanese market, Hayashibara trehalose could potentially be used to enhance the quality of a wide variety of food products.
13. The functional properties of Hayashibara trehalose could produce technical effects in foods such as cryoprotection, stabilization, flavor enhancement, color enhancement. moisture retention, non-cariogenicity, and a reduction in sweetness.

Based on its own review of the data and information supportir,g the safety of trehalose, and the recommendations of an independent Expert Panel, Hayashibara submits that there is consensus among experts (qualified by scientific training and experience to evaluate the safety of substances added to food), that Hayashibara trehalose will not be harmful under the intended conditions of use as a food ingredient.

Appendix I

Hayashibara Trehalose Expert Panel Opinion

1. Introduction

An expert panel. qualified by scientific training and experience to evaluate the' safety of food and food ingredients was assembled to conduct an independent critical evaluation of the safety of Hayashibara trehalose as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein. The panel members included:

Dr. Roy L. Whistler, Chairman	Carbohydrate Chemist
Dr. Gary Flamm	Toxicologist
Dr. Joseph Borzelleca	Pharmacologist and Toxicologist
Dr. George Burdock	Toxicologist
Mr. Cleve Denny	Microbiologist

The qualifications of the members of the Scientific Expert Review Panel (the Panel) are provided in their curricula vitae, which are on file at the offices of Hayashibara International, Inc., 2201 Civic Circle, Suite 719, Amarillo, Texas USA 75109. The Panel's qualifications in their respective scientific fields meet the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201(s)) and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

The Panel was charged by Hayashibara Company Ltd. to critically evaluate the information and data regarding trehalose in general and Hayashibara trehalose specifically. The Panel would then render an opinion, based on scientific principles, on.the generally recognized as safe (GRAS) status of Hayashibara trehalose. The opinion of the P.anel *is* based on the general use of Hayashibara trehalose in food when manufactured in accordance with U.S. current Good Manufacturing Practices (21 CFR Part 110) and meeting the specifications provided in the report.

Members of the Panel independently critically evaluated the results of a literature review on trehalose, information provided by Hayashibara, and other pertinent materials. The Panel conferred by telephone and then met in Atlanta Georgia on November 17,1999 with representatives of the Hayashibara Company, Ltd., who reviewed the pertinent safety and functionality information associated with Hayashibara trehalose. This information was compiled into an eighteen-volume report, which supported the eligibility of Hayashibara trehalose as a GRAS ingredient in accordance with 21 CFR 170.30. The information provided for the Panel's review was presented in the form stipulated in 21 CFR 170.35, and is

outlined in the Table of Contents attached as Appendix A.

The Hayashibara report included:

- A description of the chemical identity and quantitative composition of Hayashibara trehalose;
- Duplicate analyses of 5 lots of the product manufactured over approximately a six month period;
- A description of the manufacturing process and quality control program;
- A statement on the safety of the enzymes used to manufacture Hayashibara trehalose submitted by an expert in the field of enzyme safety and evaluation;
- Final product specifications for Hayashibara trehalose, and
- The physical properties of naturally occurring trehalose isolated from various sources, with a comparison to the Hayashibara trehalose product.
- The technical effects of trehalose which consisted of the following: 21 CFR §170.3(0)(4) coloring adjuncts, §170.3(0)(11) flavor enhancers, §170.3(o)(14) formulation aids, §170.3(o)(16) humectants, §170.3(0)(21), nutritive sweeteners, §170.3(0)(24) processing aids, §170.3(0)(26) sequestrants and §170.3(o)(28) stabilizers and thickeners, §170.3(o)(31) synergists, and §170.3(0)(32) texturizers.

Company representatives also reviewed:

- The historical consumption of trehalose;
- The content of Hayashibara trehalose in commonly eaten foods in Japan;
- Estimates of the current consumption of Hayashibara trehalose in Japan; and
- Estimates of the potential consumer exposure to Hayashibara trehalose in the United States.

Additionally, a nutritional analysis of trehalose was presented including:

- Data describing the physicochemical (functional) properties relevant to its use as a food ingredient, and
- Various developmental and commercial formulations containing Hayashibara trehalose were provided to demonstrate the technical effects of the ingredient in foods.

The Panel was also presented information concerning:

- The metabolism of trehalose in the human body by the enzyme trehalase;

no ADME study

- A literature review of trehalase deficiency in individuals, showing that this condition appears to be rare in western populations;
- Results of several acute and chronic toxicological studies conducted in mice, rats, rabbits, and beagle dogs;
- The published results of trehalose tolerance tests in humans.

Summaries of the key elements listed above, which the Panel used to evaluate the GRAS status of Hayashibara trehalose, are presented below in Sections 2-5.

2. Exposure

Following critical evaluation of the data and information provided by Hayashibara Company, Ltd., the Panel concluded that safe levels of exposure to trehalose could be adequately defined by limiting the use of trehalose to current Good Manufacturing Practice (cGMP) levels used commercially by Japanese food processors. In estimating exposure to Hayashibara trehalose, the Panel relied on current use data from the Japanese market place, and on USDA's *Continuing Survey of Food Intake by Individuals,* which provided daily consumption figures by major food categories for the US population [Enns, *et al.,* 1997, *Family Economics and Nutrition Review* 10: (4)]. The maximum use levels are those, which were commercialized in processed foods in the Japanese market during the years 1995-1997. The levels were reported to the Hayashibara Company, Ltd. by purchasers of the Hayashibara trehalose ingredient, or were from consumer information. Maximum use levels for the Japanese market are shown for each major food category in Table I below.

While basing their estimates of total trehalose intake on data generated by the *Continuing Survey of Food Intake by Individuals,* the panel agreed to divide the category of baked goods and other grain based products into two separate categories. This was based on the fact that a high level of use of Hayashibara trehalose (up to 20%) occurs in certain commercial rice dishes in Japan. The Japanese population consumes much larger quantities and a greater variety of rice products than do North Americans. Using a maximum use level of 20%, across all foods in the baked goods and other grain-based food category would result in an unrealistic estimate of consumption when applied to a US population. However, the fact that a high level of Hayashibara trehalose has been consumed in a food category with a high average daily intake in Japan, provides evidence that trehalose is well tolerated in that population.

The panel evaluated several.factors supporting the safe use of cGMP levels in Japan as a basis for similar use levels of trehalose in the US.

1. Hayashibara trehalose is now formulated into hundreds of food products in the Japanese market.

2. The Japanese consumer has multiple governmental and

commercial channels through which to report safety-related problems with food products. Japanese consumers are known to use these mechanisms to report food-related safety issues. The official procedures available to the Japanese consumer are described below in Section 5, Human Studies.

Since the introduction of Hayashibara trehalose to the Japanese market in 1995, in excess of 88 million pounds have been sold to the food processing industry. The Hayashibara Company, Ltd. has received no reports regarding a safety-related problem with Hayashibara trehalose.

3. Hayashibara trehalose is not expected to totally supplant currently used products for all functionalities in all food categories, as trehalose may not offer significantly greater technical advantages over other products that would offset economic considerations in many potential applications.

4. Adequate safety data exists from animal studies, published human tolerance studies, and Japanese consumption patterns to support human consumption of Hayashibara trehalose.

Based on the data and information considered, the Panel concluded that the current Good Manufacturing Practice levels for Hayashibara trehalose can be defined as those listed in Table I below.

Table 1
Current Good Manufacturing Use Levels For Hayashibara Trehalose in Foods*

Food Category[1]	Use Level[2] (maximum)
Baked Goods	10%
Rice and Other Grain-Based Dishes	20%
Vegetables and Vegetable Dishes	5%
Fruits and Fruit Preparations	5%
Milk Desserts/Cheese Products	15%
Meat, Poultry and Fish	10%
Eggs and Egg Dishes	10%
Legumes and Legume Dishes	10%
Sugars and Sweets	160%
Coffee	3%
Fruit Drinks and Ades	5%

*Based on current use levels of Hayashibara trehalose in Japan.

1. Major food categories are based on those used in USDA's *Continuing Survey of Food Intake* by *Individuals* with rice and other grain-based dishes shown as a separate category [Enns, *et al.,* 1997, *Family Economics and Nutrition Review 10:(4)].*
2. Maximum use levels are those which have been commercialized in processed foods in the Japanese Market during the years 1995-1997 as reported to the Hayashibara Company, Ltd. by purchasers of the Hayashibara trehalose ingredient.

3. **Manufacturing**

In considering the safety of Hayashibara trehalose, the Panel noted that Hayashibara Company, Ltd. has developed and patented a novel process for producing trehalose enzymatically from cornstarch. It was explained that Hayashibara Company, Ltd. was the first company in the world to commercialize the enzymatic conversion of starch to dextrose in 1959. Since that time Hayashibara Company, Ltd. has remained a leader in the enzymatic conversion of starch to many useful saccharide-based products. These products have been sold in several countries as food ingredients, and the process upon which Hayashibara trehalose is based is used by all major sweetener-producing companies around the globe. The trehalose product obtained from this process is currently approved by the appropriate regulatory agencies, and is being sold in Japan, Taiwan and Korea as a food ingredient or additive.

All raw materials used in the process are food grade in Japan, and the Hayashibara Company, Ltd. provided specifications for those raw materials in its GRAS Report. _All trehalose, whether imported or produced in the US, will be manufactured from raw materials meeting US food grade standards.

The Hayashibara technology includes the use of six enzymes, two of which are novel trehalose producing enzymes discovered by Hayashibara scientists. The following substances used in the manufacturing of trehalose are used in accordance with current Good Manufacturing Practices (21 CFR 11 O.80(a».

Activated Carbon
Alpha-amylase (EC 3.2.1.1)
Calcium Carbonate
Calcium Chloride
Corn Starch
Cyclomaltodextrin-Glucanotransferase (CGTase; EC 2.4.1.19)
Diatomaceous Earth
Glucoamylase (EC 3.2.1.3)
Hydrochloric Acid
Ion Exchange Resin
Isoamylase (EC 3.2.1.3)
·Parlite
Sodium Chloride
Sodium Hydroxide
Thermostable alpha-amylase (EC 3.2.1.1)
Trehalose Producing Enzymes
- Maltooligosyl-trehalose synthase (EC 5.4.99.15)
- Maltooli9Qsyl-trehalose trehalohydrolase (EC 3.2.1.141)

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The basic steps in the process, as presented to the Panel by company representatives, are outlined below and are essentially identical, with the exception of the two trehalose-producing enzymes, to standardized production processes used throughout the world that were originally developed by Hayashibara Company, Ltd. to produce starch-based sweeteners;

1. The production process begins with the suspension of cornstarch to produce a slurry of known concentration.

2. The pH of the slurry is adjusted, and the starch slurry is heated to a specified temperature. Liquefication of the starch is accomplished by the addition of thermostable alpha-amylase. This enzyme is inactivated by increasing the temperature of the liquefied solution. The alpha-amylase enzyme cleaves the amylose and amylopectin chains into shorter units.

3. Alpha-amylase, glucoamylase, CGTase, isoamylase, and the two trehalose producing enzymes (maltooligosyl-trehalose synthase, maltooligosyl-trehalose trehalosidase) are all added to the slurry under controlled conditions of temperature, pH and concentration. These enzymes participate in the saccharification of the liquefied starch into trehalose.

   The enzymes, with the exception of the trehalose producing enzymes, are designed to debranch amylopectin, shorten the chain length, and/or to recycle unused portions of amylose and amylopectin.

   The first of the two novel trehalose-producing enzymes, maltooligosyl-trehalose synthase recognizes the reducing end of the terminal D-glucose units of amylose molecules. This enzyme converts the alpha-1,4 linkage to an alpha-1,1 bond in an intramolecular transglycosulation reaction. An amylose molecule with the trehalose unit attached at the terminus is thus created. The second trehalose producing enzyme, maltooligosyl-trehalose trehalosidase, hydrolyses the alpha-1 ,4 bond between the second and third D-glucose unit, detaching the trehalose from the amylose molecule. The efficiency of this process is greater than 80%.

4. The trehalose-containing solution-is then decolorized with activated carbon, and the carbon and other insoluble substances are removed by filtration at a controlled temperature.

5. Other impurities, such as salts and proteins are then removed in a two-step process using industry standard twin-bed and mixed-bed ion exchange procedures

6. The purified suspension is then concentrated by evaporation.

7. The concentrated solution is further evaporated under vacuum after addition of pulverized trehalose seed, which initiates the first crystallization process.

8. The trehalose crystals are collected via centrifugation.

9. The wet crystals from initial and recycled crystallization runs are combined and dried/dehydrated in an air drier.

10. The trehalose crystals are cooled in a rotary cooler, and sized using a vibrating sieve.

11. The resulting trehalose is then packed in 20 kg units in three-ply Kraft bags, with a lot number printed on each bag. Finally, the sealed bags are screened by a metal detector.

Data was presented to the Panel, which demonstrated that all the enzymes used to produce Hayashibara trehalose were denatured during the course of processing. Of the six enzymes used, four are commercially available and have EC numbers assigned (See raw materials list above). The Hayashibara Company, Ltd. commissioned Dr. Michael Pariza, Director of the Food Research Institute at the University of Wisconsin to prepare an expert opinion regarding the safety of the novel enzymes used in the process. In his report (a letter to Dr. Alan B. Richards, dated November 14, 1999), Dr. Pariza stated "...the bacterial enzyme sources used in the production of trehalose by Hayashibara International Inc. should be considered safe and appropriate for this purpose." Dr. Pariza's report was made available to the Panel and is appended to this report as Appendix D.

The Panel critically evaluated the final product specifications for Hayashibara trehalose, and noted that the product has a high level of purity, and that the Hayashibara Company, Ltd. has well documented process controls in place. The results of analyses of 5 lots in duplicate manufactured over a period of time demonstrated that the production process is capable of meeting the defined specifications on a consistent basis. The specifications shown in Table 2 define the product in its dihydrate form.

TABLE 2
FINAL PRODUCT SPECIFICATIONS OF HAYASHIBARA TREHALOSE

Variables	Specifications
Purity (Trehalose)	≥ 98.0%
Appearance	whitish crystalline powder (dihydrate)
Coloration of the Solution	≤ 0.100
Turbidity of the Solution	≤ 0.050
pH (30% Solution)	4.5 - 6.5
Loss on Drying	≤ 1.5%
Residue on Ignition	≤ 0.05%
Lead	≤ 1 ppm
Arsenic (as AS_2O_3)	< 2 ppm
Viable Count	≤ 300 CFU/g
Coliform Organisms	negative
Yeast and Mold	≤ 100 CFU/g

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4. Safety

The Panel critically evaluated the results of several preclinical studies, which had been commissioned by the Hayashibara Company, Ltd. to assure the safety of Hayashibara trehalose. Testing included standardized *in vitro* assays, as well as oral dosing of Hayashibara trehalose to rodent and non-rodent species. Analyses of these studies consistently showed that trehalose demonstrated no oral toxicity at the levels tested. Table 3 below summarizes those results.

Table 3
Hayashibara Trehalose Safety Studies

Test/Species	Study Type	Route	Dose	Results
S. *typhimurium* and *E. coli*	Bacterial Mutation Assay	*In Vitro*	5,000 µg/plate	No evidence of mutaaenic toxicity
CHOCells	Chromosome Aberration Assay	*In Vitro*	5,000 µg/ml	No Mutagenic Activity
Micronuclear Test	Mice	IP	5,000 mg/kg	No Toxicitv
Albino Mice	Acute Toxicity (1 dose)	Oral by gavage	*5g/kg*	No Toxicity
Albino Rats	Acute Toxicity (1 dose)	Oral by gavage	*5g/kg*	No Toxicity
Beagle Dogs	Acute Toxicity	Oral by capsule	5g/kg	No Toxicity
Albino Rats	Acute Toxicity (1 dose)	Oral by gavage	*16g/kg*	No Toxicity
Beagle Dogs	14 Day	Oral by capsule	*5g/kg/day*	No Toxicity
Albino Mice	14 Day	Oral by gavage	*5g/kg/day*	No Toxicity
Albino Mice	13 Week'	Oral in diet	*8.3g/kg/day* (aver. of sexes) NOAEL*	No Toxicity
Wistar Rats	Embryotoxicityl Teratogenicity	Oral in diet	The NOAEL was the mean of the highest level tested 6.94 ± 1.61 g/kg/d	No Toxicity
New Zealand White Rabbits	Embryotoxicityl Teratogenicity	Oral in diet	The NOAEL was the mean of the highest level tested 1.99 ± *0.43 g/kg/d*	No Toxicity
Wistar Rats	Two-generation Reproductive	Oral in diet	The NOAEL was 7.09 ± 0.45 *g/kg/d* for males, and 6.16 ± *0.48* g/kg/d for females	No Toxicity

*The laboratory which performed thiS study used the term NOTEL (No-Toxlc-Effect-Level)

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After reviewing these results in a preliminary safety meeting, the Panel noted that trehalose may have multiple desirable technical effects in a number of food categories. Therefore, potential use of Hayashibara trehalose in a large variety of foods might result in higher exposure levels than was originally estimated. Therefore, the Panel recommended that additional toxicologic studies be run to investigate chronic high level exposure to Hayashibara trehalose. Accordingly, the Hayashibara Company Ltd. commissioned three studies in which the trehalose product was fed at levels up to 10% of the diet. The studies were conducted with Panel direction and essentially in accordance with the 1982 edition of the FDA handbook on *Toxicological Principles,* and current Organization for Economic Cooperation and Development (OECD) guidelines. These three studies included a two-generation reproductive study in rats, an embryotoxicitylteratology study in rabbits, and an embryotoxicity/teratology study in rats.

The individual Panel members reviewed the results of the three recent animal safety studies, which had been conducted, to satisfy this recommendation, and the results were discussed jointly during a meeting in Atlanta in November 1999.

EmbryotoxicitylTeratogenicity Study in .Rabbits and Rats

Two embryotoxicity/teratogenicity studies were commissioned by Hayashibara Company, Ltd. In the first study, 16 pregnant New Zealand white rabbits were fed Hayashibara trehalose in the diet at 0, 2.5, 5, or 10% from gestation day 0 to 29. On day 29 the dams were sacrificed and macroscopically examined. Trehalose intake during gestation ranged from 0.21 ± 0.05 to 0.77 ± 0.03, 0.48 ± 0.11 to 1.34 ± 0.11, and 1.04 ± 0.14 to 2.82 ± 0.23 grams trehalose/kg body weight/day, for the 2.5, 5, and 10% treatment groups, respectively. The results indicated that Hayashibara trehalose did not induce maternal or developmental toxicity in the offspring, and that the no-observed-adverse-effect-level (NOAEL) for this study was the mean of the highest level tested (1.99 ± 0.43 g/kg body weight/day).

In a similar study Hayashibara trehalose was fed to mated female Wistar rats (28 per group) from days 0 to 21 of gestation. The maximum consumption consumed by an animal in the 10% group during the feeding period was 5.5 to 7.8 grams trehalose/kg body weight/day. On study day 21 dams were killed and macroscopically examined. No treatment related changes were observed in any of the variables considered in the study. It was concluded that when trehalose was administered in the diet, it did not induce maternal or developmental toxicity in the offspring, even at the highest level tested. The NOAEL level for this study was the mean of the highest level fed (6.94 ± 1.61 grams trehalose/kg body weight/day).

Two-Generation Reproductive Study in Rats

A reproduction study was performed on two successive generations of male and female Wistar rats. In each generation one litter was raised. Hayashibara trehalose was added to the diet in concentrations of 2.5, 5, and 10%. The NOAEL for males during the pre-mating period was 7.09 grams trehalose/kg body weight/day. The NOAEL for females during the pre-mating, gestation and lactation periods was 7.61,6.16, and 14.09, grams trehalose/kg body weight/day respectively. The data demonstrated that trehalose had no toxic effects on: maternal variables, the reproduction of the FOand F1 parents, or on the development of the pups of either generation.

Taken individually and as a whole (See Table 3), these data confirm that Hayashibara trehalose added to the diet of 4 test species of animals (two rodent and two non-rodent) is safe up to the levels of consumption tested.

5. Human Safety Data

The Panel was presented with information which revealed that the ability to digest trehalose evolved much earlier than did hominid characteristics, and that the recorded history of human consumption of trehalose may extend to early Biblical times. The presence in the human intestinal system and kidneys of relatively large quantities of an enzyme (trehalase) that specifically cleaves trehalose into two glucose units strongly suggests that humans have the capacity to safely consume trehalose at relatively high levels. This physiological mechanism is consistent with other commonly digestible disaccharides, such as maltose or lactose, which are alsb hydrolyzed to monosaccharides prior to absorption.

It is believed that trehalose intolerance is substantially less frequent than lactose intolerance (only five cases have been reported in the literature for Western populations), although few specific studies have been performed to identify its prevalence. Information on the frequency of intolerance to isolated trehalose has historically come from three sources: human tolerance tests, enzyme assays of biopsy specimens, and recent consumption data from Japan. The Hayashibara Company, Ltd. provided the Panel with a review of the literature on human trehalose intolerance and on human trehalose tolerance tests in its GRAS Report. Noteworthy citations are summarized below and in Table 4.

It has been suggested that trehalase deficiency may occur when enzymatic activity is 6.0 IU/g protein or less, while intermediate levels would be 6.0 to 8.0 IU/g protein. Gudmund-H0yer. *et al.,* 1988 referred to a study of trehalase activity of intestinal biopsies from 248 Danish patients in which the lowest level of activity in the group was 8.3 IU/g and the median activity was 31 IU/g. The authors reported the corresponding figures for lactase activity as 6.1 IU/g and 32 IU/g respectively (Gudmund-H0yer, *et al.*, 1988 *Scan J of Gastroenterology, 23:775-778*).

In a study of 100 consecutive normal biopsy samples from adults in Switzerland (72 male, 28 female), two subjects with low trehalase activity (2.7 and 1.5 IU/g) were identified (Bergoz, *et al.,* 1973 *Scan J* of *Gastroenterology, 8:703-712).* These authors suggested that a trehalase value < 5 IU/g might result in intolerance of trehalose ingestion. This conclusion is in basic agreement with that of Gudmund-H0yer, *et al.,* 1988 (Gudmund-H0yer, *et al.,* 1988 *Scan J of Gastroenterology,* 23:775-778). It is important to note that low values for enzyme activity are highly correlated, but not an absolute predictor of disaccharide intolerance (Dahlqvist, 1974 Enzyme Deficiency and Malabsorption of Carbohydrates, (Ed.) H. Sipple, (Pub.) Sugars in Nutrition, Academic Press, New York, pp. 187-217).

A review of the available literature showed that individuals in western populations consumed at least 50 grams of trehalose in a bolus without reporting ill effects.

No populations with a high incidence of trehalase deficiency had been described prior to the work of Gudmand-Heyer in 1988. In their study 97 Greenlanders who had undergone operations for peptic ulcer or cancer of the stomach or pancreas were biopsied. Eight of the patients had a trehalase activity of less than 6 IU/g and 14 had an activity of less than 8 IU/g. However, no trehalase deficiencies were identified in more than 500 biopsies of Danish subjects (Gudmund-H0yer, *et al.,* 1988 *Scan J of Gastroenterology, 23:775-778).*

Welsh described intestinal trehalase activity in 123 Caucasian subjects from the southwestern United States, ranging in ages from 1 month to 93 years (Welsh, *et al.,* 1978 *Gastroenterology,75:847-855).* The lowest recorded values were 7 and 8 *IU/g* in two groups of infants 0 to 1, and 1 to 2 years of age (n=70), respectively. No statistically significant differences in trehalase activity were found between any age group or by gender. Importantly, trehalase activity did not appear to wane with age.

Twenty patients in Czechoslovakia with no bowel symptoms or disease were examined for trehalase activity (Madzarovova-Nohejlova" *et al., 1973 Gastroenterology,* 65: 130-133). None of these subjects were considered to be trehalase deficient.

Bergoz, 1971, tested 16 control subjects for their ability to assimilate trehalose using a tolerance test very similar to that used for glucose (Bergoz, 1971, *Gastroenterology,* 60(5): 909-912). Each subject drank 50 grams of glucose in water, followed within 2 days by a similar preparation of trehalose. Blood glucose values were assayed after ingesting glucose or trehalose and the ratios of glucose absorbed were calculated. All control subjects tolerated both test solutions and assimilated the glucose hydrolyzed from trehalose (ratio = 0.70, range 0.31-1.42); however, the time to peak blood glucose concentrations was slower after trehalose ingestion. In another study Bergoz *et al.* reported on 50 hospitalized control subjects (Bergoz, *et al.,* 1973 *Scan J of Gastroenterology,* 8:657-653). Patients were given glucose and trehalose oral tolerance tests as described above. Control subjects had a median absorption ratio of 0.70 (range of 0.31 to 1.52) and tolerated the trehalose exposure. These values were essentially the same as in the original study.

Bolte, *et al.,* 1973 performed trehalose tolerance tests on 60 German control patients without gastrointestinal disease. No trehalose intolerance was observed (aolte, *etal.,* 1973, *Dtsch. Med. Wachr., 98:1358-1362).*

Table 4
Human Trehalase DeficiencylTolerance Studies Cited

Subject Numbers	Location	Results
500 Biopsies	Denmark	No trehalase deficiencies observed
33 Biopsies	Oklahoma	Trehalase normal even in 7 lactase deficient subjects; Children similar to adults; Low in 3 celiac disease, but similar to other enzymes
123 Biopsies	Southwestern U.S.	No Trehalase deficiencies. Two neonates had low levels; No significant differences between age groups; Enzyme activity did not diminish with age
13 Biopsies	Denmark	No trehalase deficiencies observed
100 Biopsies	Switzerland	Two with low trehalase activity
20 Control Patients	Czechoslovakia	No trehalase deficiencies observed
16 Control Patients	Switzerland	Tolerated 50 grams trehalose
50 Control Patients	Switzerland	Tolerated 50 grams trehalose
60 Control Patients	Germany	Tolerated 50 grams trehalose

Out of more than 700 biopsies reported, only two were shown to be deficient in trehalase and two had levels that would be considered intermediate (6 IUfg protein or less). Of 146 control patients given 50 grams of trehalose in a bolus on an empty stomach, none were reported to be intolerant. Taken together, it appears that when trehalase activity assays or trehalose absorption tests are performed on hundreds of control subjects from a western Caucasian population, only a few individuals could be identified with low trehalase activity. Importantly, this percentage « 1%) appears substantially less than lactase deficiency and possibly other disaccharidase deficiencies (Dahlqvist, 1974 Enzyme Deficiency and Malabsorption of Carbohydrates, (Ed.) H. Sipple, (Pub.) Sugars in Nutrition, Academic Press, New York, pp. 187-217).

The Panel noted that malabsorption and/or intolerance to trehalose has been reported, and that clinical symptoms appear to be identical to those seen in other disaccharide malabsorption syndromes, and are therefore self-limiting.

The first suggested case of trehalase deficiency was reported by Bergoz in 1971 in a 71 year old Swiss woman (Bergoz, 1971, *Gastroenterology,* 60(5): 909-912). She had noticed for at least 20 years that mushrooms provoked diarrhea. In order to test the hypothesis that this woman was lacking trehalase, the author conducted a study in which patients were given 50 grams of glucose in 400 ml of water. Blood samples were taken at intervals between 0 and 120 minutes. During the trehalose tolerance test the patient intolerant of mushrooms suffered bloating, abdominal cramping, and presented liquid stools starting 70 minutes after trehalose was ingested. Trehalose absorption tests were conducted on this patient and 16 controls. The mean absorption ration[4] for trehalose to glucose was 0.02 for the patient intolerant to mushrooms, and 0.70 for the 16 controls.

Madzarovova-Nohejlova in a 1973 report discussed the case of a 24-year old white man in Czechoslovakia who was admitted to University Hospital with vomiting and diarrhea resulting from the ingestion of mushrooms. It was later discovered that other members of his family also experienced mushroom intolerance. A trehalose tolerance test was performed after a 50 mlload was administered. Complete absence of trehalase activity was found in the patient and his father. The conclusion was that an autosomal type of heredity for trehalase deficiency seemed likely (Madzarovova-Nohejlova, *et al., 1973 Gastroenterology,* 65: 130-133).

Two recent studies from Japan provided data on trehalose absorption in humans. Thirty subjects (15 female, 15 male) included 10 Mongoloid (Japanese), 10 Caucasians, 8 African-American, and 2 designated as other (Ushijima, *et al.,* 1995 Digestion and Absorption, 18: 56-57). Subjects were fasted overnight and the subjects were given 10, 20, 30 and 40 gram doses of trehalose. Blood glucose concentrations and hydrogen (or methane) gas expiration was measured before and every 30 minutes after for 3 hours. An increase of 20 ppm of hydrogen (or 1 ppm methane) was considered to be a sign of malabsorption. Malabsorption rates of 0, 40, 43 and 75% were observed when subjects ingested 10,20,30 and 40 grams, respectively. Gastrointestinal (GI) symptoms were also observed, with occurrences of 0, 40, 43, and 50%, respectively. No breakdown was reported as to the response according to racial groupings. The severity of the malabsorption or GI symptoms was not noted.

In a second experiment using the same group of subjects, 0.6 grams of trehalose per kg body weight was administered. The differences in malabsorption rates were not significant between the racial groups (Mongoloid =50%, Caucasian = 67%, African-American =63%). Conversely, the Monogoloid subjects had dramatically higher GI symptoms (90%) than either the Caucasian (11 %) or

African-American (0%) subjects. Blood glucose concentrations (30 minutes) were significantly higher in Caucasians (37.6 ± 11.1 mg/dl) and African-Americans (24.3 ± 10.3) than Mongoloid subjects (11.0 ± 8.6). These data suggest that in this study the administration of 20 grams or greater resulted in malabsorption (reduced absorption) of trehalose. When equal amounts of trehalose are given on a body weight basis, Caucasians and African-Americans appear to be able to absorb glucose at a significantly higher rate than Mongoloid subjects. This is not consistent with the data showing similar malabsorption using the expiratory method in which Mongoloid subjects showed lower malabsorption.

A trehalose and lactulose tolerance study was performed on healthy Japanese women (Oku, *et al.,* 1998 J Nutr, Sci. Vitamonol. 44:787-798). Doses of 30,40, 50 and 60 grams of trehalose in 200 ml water were given to each sUbject 2 to 3 hours after eating. Subjects ingested the next greater amount of saccharide until they experienced diarrhea. Subjects recorded the time of onset and type of abdominal symptoms (if any), and stool frequency and consistency. No subjects had diarrhea when given 30 grams of trehalose. At 60 grams of trehalose, 10 of the 20 subjects had reported diarrhea. Abdominal symptoms were reported in subjects at the 30 gram dose. These included: nausea (10%), discomfort (15%), flatus (40%), distension (20%), borborygmus (45%), and *low* abdominal pain (5%). The symptoms became progressively more prevalent as the dose increased. The severity of each symptom was not recorded. The authors calculated the transitory laxative threshold of trehalose as 0.65 glkg body weight using regression analysis (0.28 glk:g for lactulose). This calculates to a dose of 33 grams for a person weighing 50 kg, which was the average of the study popUlation. The authors made the point that this study used a single large dose of trehalose, given as a bolus. It has been shown that splitting doses between several eating occasions can increase the ability of the system to digest similar substances. It is known that when disaccharides (trehalose) are used in a food product they are digested over a longer time period, thus allowing for more *complete* digestion *(Elias, et al.,* 1968 *J Physio/., 194:317-326).*

It appears from the data presented that Mongoloid populations may not have as great a capacity to absorb trehalose; however, the amounts that are digestible appear to be relatively large. In a total of 30 Mongoloid subjects a single dose of 30 grams was well tolerated. In the 146 subjects from a predominately Caucasian population given a single dose of 50 grams of trehalose, no subjects reported abdominal problems. The first Japanese study showed that no abdominal symptoms were reported by the 10 African-Americans and only 1 of 9 Caucasians given 0.6 *g/kg* body weight of trehalose; the equivalent of 42 grams of trehalose for a 70 kg man reported symptoms. Ingesting trehalose at multiple times during a day, and including it in food products would likely raise the no effect dose level of trehalose considerably above the amounts suggested in these studies.

6. Conclusion

The Expert Panel independently and collectively reviewed and critically evaluated all the data and information summarized in the Opinion given above. It was unanimously determined that Hayashibara trehalose meeting appropriate food grade specifications and manufactured with the use of safe enzymes is generally recognized as safe (GRAS) by scientific procedures, when used as a multipurpose food ingredient in accordance with cGMP. Uses and use levels in Japan provide the basis for establishing cGMP.

Signatures of Expert Panel Members:

Dr. Roy Whistler, Chairman

Date 05/09/00

Dr. Joseph Borzelleca

_____ Date _____

Dr. George Burdock

_____ Date _____

Mr. Cleve Denny

_____ Date _____

Dr. Gary Flamm

_____ Date _____

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Signatures of Expert Panel Members:

Dr. Roy Whistler, Chairman

_____ Date _

Dr. Joseph Borzelleca

Date 08 May 2000

Dr. George Burdock

_____ _____

Mr. Cleve Denny

_____ Date _

Dr. Gary Flamm

_____ Date. _

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Signatures of Expert Panel Members:

Or. Roy Whistler, Chairman

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Dr. Joseph Borzelleca

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Dr. George Burdock

[redacted] Date 18 May 00

Mr. Cleve Denny

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Or. Gary Flamm

[redacted] _____ Date 12 May 00

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Dr. Joseph Borzelleca

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Dr. George Burdock

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Mr. Cleve Denny

Date 5/9/2000

Dr. Gary Flamm

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Appendix A

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Appendix B
Japanese Consumer Complaints

There are several methods in Japan by which consumer complaints concerning food products can be reported. The various government agencies to which people can report include: the Japan Consumer Information Center (JCIC); Public Health Centers; Prefectural Government Offices (e.g. Tokyo Metropolitan Government, Bureau of Citizens and Cultural Affairs, Consumer's Center); Economic Planning Agency (Social Policy Bureau); and Ministry of Agriculture, Forestry and Fisheries (Consumer Life Division). The first three are the most common contact points for consumer complaints, and it is likely that any consumer inquiry to a central government agency would be referred to JCIC. The JCIC is a nonprofit organization which was established by the Japanese Government in 1970 to provide consumer education, training programs and publications, test products, alert consumers to potential problems with products, and handle consumer complaints.

The process by which JCIC handles complaints includes collecting the appropriate information from the consumer, contacting the company, reviewing the available information, formulating a response, implementing any responses or corrective measures which could include working with the company(ies) involved or industrial organizations, and the dissemination of pertinent information to relevant government authorities. If companies are not responsive to the concerns of JCIC, the case is turned over to appropriate agencies for legal action.

In addition to government associated agencies, manufacturers encourage consumer comments directly to them and usually provide a telephone contact number on their product. Since trehalose is usually used as an ingredient in a food product, it is more likely that any complaints would first be made to the final product manufacturer or to the JCIC. SUbsequent to a complaint, Hayashibara would be contacted if there was a question of whether trehalose was involved. Additionally, a subsidiary of Hayashibara, H+B Life Science, has sold a tabletop sweetener containing 100% trehalose for more than one year and has access to direct customer comments.

Although tens of thousands of metric tons of Hayashibara trehalose have been sold into the Japanese market since the end of 1995, no consumer complaints have been reported to either Hayashibara Shoji (bUlk sales), the R&D Center (Regulatory Affairs), or H+B Life Science. This provides strong evidence that consumption of trehalose is safe and has not produced untoward effects in the concentrations being used in the
Japanese food industry.

Appendix C
Salient points
Hayashibara GRAS Report

The Panel also wished to acknowledge the salient points of the Hayashibara Company, Ltd. GRAS Report, which had been presented at an earlier meeting. They appear below as Appendix C.

Section I. Chemical Identity of Trehalose

- Trehalose is a stable non-reducing disaccharide with two glucose molecules linked in an a, α 1,1 configuration
- Specifications for raw materials are food grade in Japan
- Analyses of 5 lots in duplicate manufactured from November 16, 1996 to March 3, 1997 showed that the trehalose manufactured by Hayashibara Company Ltd. was of high purity, and fell within the parameters of its food grade specifications
- The Hayashibara manufacturing process as outlined included critical control points to ensure the consistency and safety of the product
- Adequate processes and controls are established for the isolation procedures used in recovering the novel enzymes, maltooligosyl-trehalose synthase and maltooligosyl trehalose trehalosidase from the soil bacterium *Arthrobacter ramosus.* This naturally occurring nonpathogenic and nontoxigenic gram-positive rod shaped soil bacterium is a member of the Genus, *Arthrobacter.*
- The chemical specifications set for trehalose meet food grade standards
- A tracking program is established in the event a product recall is necessary
- Various physical properties of trehalose were described including its crystalline form, its structural analyses, its melting point, and its specific optical rotation
- Trehalose exists naturally in plants (sunflower seeds); food (honey, wines, Sherries, invert sugars, Mirin, breads and mushrooms); invertebrates (lobster, crabs, prawns, brine shrimp, and insects); humans; fungi; yeast; and other microorganisms.
- Hayashibara trehalose was shown to be identical to naturally occurring trehalose isolated from various species

Section II. Use and Functionality

- Commercial use of trehalose began in Japan in January 1995 and has continued to expand in this market
- Nutritional analyses of Hayashibara trehalose show that the energy available from the sugar equals 362 Kcal/1 DOg
- The technical effects of trehalose in food were shown to be related to its mechanisms of action in nature, including substituting for structural water by forming hydrogen bonds with macromolecules, protecting tertiary structures of proteins and phospholipids by suspending them in an amorphous glassy solid

Appendix C Continued

- and preserving the stability of nutrient macromolecules by remaining chemically inert
- A taste test panel concluded that trehalose had a sweetness value of 45 In comparison to sucrose, with a value of 100
- Various physiochemical properties related to food applications included pH stability, heat stability, heat stability in the presence of proteins, and storage stability in solutions
- Trehalose is documented by published and unpublished literature to have the following technical effects in food; cryoprotectant, stabilizer, flavor enhancer, color enhancer, moisturizing agent, and noncariogenic sweetener
- Published literature provides evidence of significant trehalose consumption by ancient man
- No significant consumption of isolated trehalose has occurred in the United States prior to 1958
- Approximately 88,000,000 pounds of Hayashibara trehalose have been sold into the Japanese market since its introduction in 1995.
- Trehalose is expected to be consumed as a macronurtient in food products
- Uses of trehalose in commercial foods ranged from 0.5% to 65.2% of certain products
- Trehalose is shown to be metabolized by the enzyme trehalase which, in humans is located in the brush border of the intestinal tract and in the renal proximal tubules of the kidney
- Trehalose was shown to be rapidly metabolized into glucose by both humans and animals
- Trehalase, levels are fairly constant in humans and no age or sex differences have been reported in the literature
- Tolerance studies in humans show that greater than 98% of consumers produced the enzyme necessary for normal metabolism of trehalose
- Trehalose has been shown to be both manufactured and hydrolyzed in the human kidney
- Rare trehalase deficiencies have been reported in isolated ethnic populations and in three European individuals
- At risk populations include long term diabetics, and persons with liver disease, rheumatoid arthritis or malabsorption diseases

Section III. Analytical Methods

- An analytical method based on gas liquid chromatography provides for the identification of trehalose in food

Appendix C Continued

Section IV. Safety

- Various acute studies in mice, rats, and beagle dogs indicated that there were no clinically observable effects from the consumption of trehalose at levels up to 5 g/kg per day [Lethal doses of trehalose were shown to exceed 16 g/kg in rats]
- A 90-day oral toxicity study showed that mice consuming the human equivalent of 100 grams of trehalose per day showed no clinically observable effects
- A two-generation reproductive study in rats and embryotoxicity/teratology studies in rabbits and rats have shown that the no-observed-adverse-effect-level, (NOAEL) for reproductive effects after dietary administration of trehalose to rats was at least 7.09 g/kg for pre-mating males, 7.61 g/kg for pre-mating females, 6.16 g/kg during gestation, and 14.09 grams of trehalose/kg body weight/day during lactation -
- Safe levels-of consumption derived from various studies in animal models translate into consumption levels ranging from 72.8 to 986.3 grams per day for a human weighing seventy (70) kilograms
- Japanese/Mongoloid populations may be more sensitive to trehalose malabsorption than Caucasian or African American populations
- No consumer food-safety related complaints have been received by the Hayashibara Company, Ltd., since the introduction of the product in 1995
- Japanese consumers have a procedure available to them for reporting food-safety related incidents
- Trehalose has been shown not to lower oral pH below 5.2, suggesting that trehalose does not contribute to dental caries
- High doses (up to 5,000 μg/ml) of trehalose were shown to have no deleterious effects on chromosomes
- The United Kingdom approved trehalose as a novel food in the early 1990s

Section V. Environmental Assessment

- The Hayashibara Company Ltd. provided an environmental assessment document that shows that the manufacture of trehalose poses no threat to the environment

AppendixD

Enzyme Expert Opinions

Michael W. Pariza Consulting, LLC
7102 Valhalla Trail
Madison, WI 53719-3039

Michael W. Pariza, Member

November 14, 1999

Alan B. Richards, Ph.D.
Vice President
Hayashibara International Inc.
2201 Civic Circle, Suite 719
Amarillo, TX 79109

FAX: 806/468-7712

Dear Dr. Richards:

I am writing in regard to the bacteria used in the production of trehalose by Hayashibara International Inc., My opinion is based on material that you provided as well, as publicly-available information relating to the safety and suitability of these microorganisms for food enzyme manufacture.

Conceptually such bacteria may be thought of as *enzyme suppliers*, analogous to companies that supply, manufacturing ingredients. In assessing ingredients that are purchased for food manufacture it is common practice to require that GMPs have been adhered to, and to establish "appropriate" specifications for composition including, potential contaminants. In the same way one should utilize "appropriate GMPs for food enzyme manufacture from microbial hosts, including appropriate product specifications that consider potential contaminants.

In the case of microbial enzymes, the sources of potential contamination are the ingredients that comprise the growth media, and the microorganisms themselves. Both require careful monitoring. The potential contaminants from the growth media are assessed in the final product that is used in food processing, again via the development of appropriate specifications for the product. The potential contaminants from the microbial source organisms are assessed in similar fashion (tests for specific microbial toxins, for example mycotoxins, where appropriate). In addition the appropriateness of a

microbial enzyme source is assessed via the concept of *safe strain lineage*.

There are several reasons for concluding that the bacteria used in trehalose manufacture by Hayashibara International Inc. are safe and appropriate for this purpose. The bacterial species to which these strains belong have not been linked to food poisoning episodes. There is no evidence of enterotoxin production or pathogenicity by the bacterial species to which these strains belong. The animal feeding tests conducted by Hayashibara indicated no evidence of adverse effect,' Finally, it should be noted that the bacteria used in the production process, including Hayashibara's proprietary strain of *Pseudomonas amyloderamosa*, are deposited with the America.n, Type Culture colleeeion (ATTC). Hence, these microorganisms are available for non-commercial use by FDA, academic, and, industry scientists who may for example wish to independently investigate their biochemical characteristics including appropriateness for use in food ingredient manufacture.

Accordingly, in my opinion, the bacterial enzyme sources used in the production of trehalose by Hayashibara International Inc. should be considered safe and appropriate for this purpose.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of your product by you or others.

Sincerely,

Michael W. Pariza, Ph.D.
Wisconsin Distinguished Professor
Member, *Michael W. Pariza Consulting LLC*

CLEVE DENNY
Food Consultant
6230 Valley Road
Bethesda, MD 20817
(301) 365-4581

Microbiology
Food Technology
Regulations
(NFPA Retired)

November 17, 1999

Hayashibara Trehalose GRAS Opinion

I have examined all of the relevant information provided to me regarding the safe use of Hayashibara trehalose in food. Additionally, I have consulted other references with which I am familiar. Several of those I have discussed below. Based on my review of these materials it is my considQred opinion that Hayashibara trehalose be considered a GRAS food ingredient limited only by Good Manufacturing Practices.

I was asked to review the organisms, which are the source of several of the enzymes used by ttayashibara in the production of Trehalose. During my review, I provided to Dr. Alan Richards of Hayashibara International, *the Partial List of Enzyme Preparations That* Are *Used* in *Foods.* On November 6, I also sent *The "Bad Bug Book",* both of which are published by FDA. Note that nothing in either publication indicates that any of the organisms identified with the manufacture Trehalose are condemned.

In fact, *Aspergillus niger* is allowed to produce carbohydrase, cellulase, glucose oxidase-catalase, pectinase, and lipase, so I do not believe FDA could object to its use for other catalytic reactions.

Also, *Bacillus licheniformis* is approved for carbohydrase and protease enzyme, so there should be no objection there.

BacillUS subtilis. . The USDA Western Regional Laboratory in 1950 was proposing that subtilin (an antibiotic) produced by *Bacillus subtilis* be used to prevent spoilage in foods_ It was later found not to be completeiy effective for that purpose, but certainly is not toxigenic. It is allowed to produce carbohydrase and protease by FDA.

Bacillus circulans is not listed in the *Bad Bug Book* as a pathogen. It is not listed in Toxline and the Toxic Data Base at

the National 'Library of Medicine. This is a common bacillus and much work has been done on the enzymes cyclodextrin glycosyltransferase and cyclornaltodextrin glycanotransferase that it produces. I do not believe that it would be rejected for use in manufacturing Trehalose, particularly given the results of the animal feeding studies that I examined.

Bacillus cereus, which can be a food poisoning organism, is allowed to produce milk clotting enzymes, so I see no problem with the non-pathogen *Bacillus circulans* and *B. subtilis* being used.

Arthrobacter ramosus is a common soil borne organism which bas been, found to be nickel-resistant. It is not considered a pathogen in the Bad Bug Book, or in Toxline or the Toxic Data Base. I could find no bad effects associated with it in the data to date.

Pseudomonas *amyloderamosa* and its enzyme isoamylase have been studied in detail by Y. Katsuya, Y. Mezaki, M. Kubota, and Y. Matsura. The organism is widely distributed in water and soil. It is not listed in the Bad Bug Book as a food pathogen and neither is any other Pseudomonas. However, one strain, *Pseudomonas aeruginosa (pyocyanea)* is considered pathogenic under certain circumstances in man. More than likely, this latter strain is so far unrelated to *Pseudomonas amyloderamosa,* that FDA will not raise any questions as to the safety of the enzyme.

As a GRAS Panel member, after reviewing the world references on the organisms and their enzymes, after studying the three newly completed animal studies and the executive summary that was provided to me, I cannot believe that Trehalose as produced by the procedures outlined by Hayashibara International would constitute any health problem, even if consumed in fairly large amounts over a long period of time.

Sincerely,

Cleve B Denny

Appendix II

Mich--l W. Pariza Consulting, LLC
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Cleve a Denny

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Appendix III

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To: Linda S. Kahl, Ph.D.
Food and Drug Administration
Center for Food Safety &Applied Nutrition
HFS-206, 200 C Street SW
Washington, D.C. 20204

Date: May 18.2000
Pages: 2
Tel: 202-418-3101
Fax: 202-418-3131

From:Alan B. Richards, Ph.D.

Dear Dr Kahl:

Please find on the second page the letter that we discussed on our telephone conversation of May 17,2000. The statement was really truly omitted. As you can see for the old second sentence. "However, throug,hout (etc.)" it appears that there is a sentence missing immediately prior to this. Both must have been deleted somehow. Anyway, please note the wording in the Jetter and I hope that this satjsfies the Agencies request.

One additional matter, that I believe Lee Dexter mentioned to you, Our company would greatly appreciate the posting of the reception of our Notification submission on the internet site at your earliest possible convenience.

Thank you again for your help in these matters.

Sincere1y,

Alan B. Richards
Vice President

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May. 18, 2000

Original to follow

Linda S. Kahl, Ph.D.
Food and Drug Administration
Center for Food Safety and
AppHed Nutrition
HFS-206, 200 C Street SW
Washington, D.C. 20204

Dear Dr. Kahl:

Thank you for your telephone call of May 17.2000. In reviewing the GRAS Notification submission dated May 3, 2000 titled *"Hayashibara Trehalose"*, it was discovered that an inadvertent omission was made in item 7 on page 3. The document states: "The data and information that are the basis of the GRAS determination for Hayashibara trehalose will be available at the address of the notifier listed above." It should have read 'The data and information that are the basis of the GRAS determination'for Hayashibara trehalose will be available for FDA review and copying at the address of the notifier listed above. The notifier will also be pleased to provide the FDA With a copy of the GRAS report, or any refere,nces contained therein. upon written request."

An additional correction should be made in the next sentence to clarify the meaning. The word "However," should be deleted and the sentence should read "Throughout this Notification, citations to the published literature, which were included in the 18-volume GRAS Report are denoted as follows: [Author *(et al.)*, Year, Tab (number) Volume (number)]."

Thank you for your initial review, comment, and consideration in this change. Please feel free to contact me at any time if you have additional comments or questions about the submission.

Sincerely.

Alan B. Richards, Ph.D.
Vice President

copy: Mr. Katsuaki Hayashibara
Ms. Lee B. Dexter

000222

[redacted]

Submission End

[redacted]

000223